## R E M A R K S

The Office Action dated August 29, 2003, presents the examination of claims 6, 7, 9, and 12. No amendments are made to the application and thusly no new matter is inserted into the application.

***Interview***

An interview was held with the Examiner at the United States Patent and Trademark Office on January 15, 2004. The Examiner's assistance in advancing prosecution of the present application is greatly appreciated.

***Request for Telephonic Interview***

If, for any reason, the instant Reply does not place the present application into immediate condition for allowance, the Examiner is respectfully requested to contact Kristi L. Rupert, Ph.D. (Reg. No. 45,702) at 703-205-8000 prior to the issuance of a Final Office Action.

***Rejections under 35 U.S.C. §§ 101, 112***

The Examiner maintains the rejection of claims 6, 7, 9, and 12 under 35 U.S.C. §§ 101 and 112, first paragraph, for allegedly not

being supported by either a credible, substantial or well-established utility. Applicants respectfully traverse. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

In order to prove that the present invention has a credible, substantial or well-established utility, Applicants submit herewith Exhibits 1 and 2, which show that a clinical trial has been conducted in **human patients** and that the present invention is practically useful in the real world.

On page 3, lines 13-16 of the Office Action, the Examiner states:

> *"[T]he art of Yamana et al is not persuasive because the reference is only in English for the abstract and in the abstract it states that the* ***phase I trial is in vitro*** *and* ***only works in some cases*** *and* ***it is not clear from the abstract what cases or conditions were studied*** *[emphasis added]."*

Applicants respectfully disagree with the Examiner's assertions and will address each assertion in turn.

***"phase I trial is in vitro"***

With regard to the Examiner's assertion that the trials are in vitro, as can be readily seen from the English translation of H.

Yamana and K. Itoh, *Jap. J. Cancer Chemother.*, 27(10):1477-1488 (2000) (attached hereto as Exhibit 1), the "phase I trial" in question is an in vivo clinical trial for human patients and is by no means an "in vitro" trial. The Examiner might have been misled by the description of "in vitro study" at line 9 of the Abstract of Yamana et al. However, from the context of the paragraph, it is clear that *blood collected from a patient undergoing a phase I trial* was subjected to an *in vitro* test for evaluation. However, the patient underwent an in vivo phase I trial.

***"it is not clear from the abstract what cases or conditions were studied"***

In order to show the cases and conditions of the clinical trial, Applicants submit herewith Mine et al., "Interim Analysis of Cancer Peptide Vaccine Phase I Test," *Biotherapy* 15(3):181 (2001) and English Translation thereof as Exhibit 2. The article shows that (1) a phase I trial for SART-1 derived peptide was started in May of 1999; (2) the subjects of the phase I trial for SART-1 are esophageal/lung squamous cancer patients; (3) the dosage schedule was 0.3 mg/body, 1.0 mg/body and 3.0 mg/body; (4) the case number is 3 to 4; and (5) induction/enhancement of CTLs were observed due to administration of the peptides.

***"only works in some cases"***

The Examiner further asserts that that since the peptides are expressed in both normal and cancerous cells, there is no "specific" utility. Applicants respectfully submit that the Examiner is improperly maintaining the lack of utility rejection against established case law. In order to satisfy 35 U.S.C. § 101, a claimed invention does not lack utility merely because the particular embodiment disclosed lacks perfection. In other words, an invention that is only partially successful in achieving a useful result is sufficient. See In re Brana, 51 F.3d 1560 (Fed. Cir. 1995); In re Gardner, 475 F.2d 1389 (CCPA 1973); In re Marzocchi, 439 F.2d 220 (CCPA 1971). Thus, the Examiner's statement that the results of Yamana show that the peptides "only work in some cases" actually proves that the claimed invention, albeit only allegedly partially successful, achieves a useful result.

For all of the above reasons, the rejection under 35 U.S.C. §§ 101 and 112, first paragraph is improper. Withdrawal of the instant rejection is therefore respectfully requested.

***Rejection under 35 U.S.C. § 112, first paragraph, enablement***

The Examiner rejects claims 6, 7, 9, and 12 under 35 U.S.C. § 112, first paragraph for an alleged lack of enablement. Applicants

respectfully traverse the rejection. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

On page 4, last paragraph of the Office Action, the Examiner states:

*"The claims are broadly drawn to a tumor antigen and peptides of such that the peptides bind HMC [sic] I antigen and are recognized by CTLs."*

The Examiner's statement is clearly incorrect. The protein of claim 6 is defined by an amino acid sequence (SEQ ID NO: 2) and a DNA (SEQ ID NO: 1) encoding the same. Similarly, the tumor antigen peptide of claim 7 is clearly defined by a partial sequence (e.g., 749-757) of SEQ ID NO: 2. Thus, the claims are not "broadly drawn" to any tumor antigen and protein. Further, a method for evaluating the activity of the present tumor antigen protein to bind MHC I antigen and be recognized by CTLs is described specifically in the specification so that a skilled artisan can readily carry out the invention without the need for undue experimentation.

The Examiner also relies on Ezzell (1995), Spitler (1995) and Boon (1992) to assert that cancer therapy is an unpredictable field of art, and thusly the skilled artisan could not predict whether or not the claimed peptides would function as successful vaccines.

According to the Examiner, Spitler states "cancer vaccines don't work." (See page 5, line 16 of the Office Action).

Applicants respectfully submit that the Examiner's comments are not justified. In fact, several tumor antigens have been found prior to the priority date of the present application. Further, several articles have been published showing the clinical effectiveness of tumor antigen peptides. Slingluff et al., *Clinical Cancer Research*, 7:3012-3024 (2001) (attached hereto as Exhibit 3) describes the successful phase I trial of a melanoma cancer vaccine with the $gp100_{280-288}$ peptide. Rosenberg et al., *Nature Medicine*, 4(3):321-327 (1998) (attached hereto as Exhibit 4) also describes the use of gp100 peptides for the treatment of melanoma. Marchand et al., *Int. J. Cancer*, 80:219-230 (1999) (attached hereto as Exhibit 5) describes the successful treatment of patients with metastatic melanoma with MAGE-3.A1 peptide.

The predictability of efficacy of a certain cancer vaccine should be evaluated individually and not generally. It is well known in the art that many researchers are involved in the investigation and development of cancer antigens, which fact strongly suggests that a cancer vaccine is a promising strategy for treating and preventing cancer. As noted by Rosenberg et al., "Synthetic peptide vaccines based on the genes encoding cancer

antigens hold promise for the development of novel cancer immunotherapies." (See abstract). Thus, it is not reasonable for the Examiner to conclude that a cancer vaccine is useless or unpredictable, when those skilled in the art clearly value cancer vaccines.

On page 6, line 6 to page 7, line 10, and also on page 8, second paragraph of the Office Action, the Examiner discusses DNA vaccines. As discussed during the interview held on January 15, 2004, the instant claims are not drawn to a DNA vaccine. Thus, the Examiner's remarks are irrelevant and should be withdrawn from the record.

On page 7, line 11 the Examiner asserts, "[T]reatment of cancer in general is at most unpredictable…." Similarly, on page 8, lines 3-4 of the Office Action, the Examiner asserts, "[I]t would require undue experimentation by one of skill in the art to practice the invention as claimed."

The Examiner is reminded that is no requirement for human clinical trials to prove that the cancer vaccines will work. "Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials." U.S. Pat. & Trademark Off., Manual Pat. Examining Proc. § 2107.03 IV (8th ed., Rev. 1, Feb. 2003). Nevertheless, Dr. Itoh (an inventor of the

subject matter of the present application) has entered clinical trials for the tumor antigen peptides, documentation of which is shown in Exhibit 2, discussed above.

> "In order to determine a protocol for phase I testing, the first phase of clinical investigation, some credible rational of how the drug might be effective or could be effective would be necessary. Thus, as a general rule, if an applicant has initiated human clinical trials for a therapeutic product or process, Office personnel should presume that the applicant has established that the subject matter of that trial is reasonably predictive of having the asserted therapeutic utility."

U.S. Pat. & Trademark Off., Manual Pat. Examining Proc. § 2107.03 IV ($8^{th}$ ed., Rev. 1, Feb. 2003) (emphasis in original). Dr. Itoh is a Professor of Medicine at the Medical School of Kurume University and supervises the clinical trial, which was initiated once the ethical committee of his University gave approval. Since Dr. Itoh has already entered phase I clinical trials and has already provided a convincing rationale that the investigation may be successful, the Examiner's insistence on maintaining the rejection of the instant claims is improper.

During the interview, the Examiner reiterated that the specification does not show differential expression of the SART-1 peptide and asserted that there is no evidence currently of record showing that the inventive peptides work. Applicants respectfully submit that the Examiner's reliance on the expression pattern of SART-1 in asserting that the inventive peptides would not function is improper. One of skill in the art would never presume that a tumor antigen peptide would not work simply based on its equal expressed in both cancerous and non-cancerous tissues for the following reasons:

(1) As described in the instant specification, SART-1 of the present application is expressed in various normal tissues at the mRNA level; however, as shown in Table 3 of Exhibit 1, the expression is unevenly distributed to cancerous cells and tissues at the protein level. That is, the expression is mainly found in cancerous cells and tissues at the protein level. This fact suggests that the expression of SART-1 must be controlled at the stage of translation of mRNA into protein; and

(2) At page 6-7 of Renkvist et al., *Cancer Immunol. Immunother.* 50:3-15 (2001) (attached hereto as Exhibit 6), there can be found various tumor antigens in addition to SART-1 that are

expressed in both cancerous and normal cells. See, "Table 3: Class I HLA-restricted widely expressed antigens."

Accordingly, in general, the fact that a tumor antigen is expressed in both cancerous and normal cell lines does not necessary mean that the tumor antigen is unavailable as a treatment for cancer. Further, given the success of clinical trials conducted by Dr. Itoh, it is clear that the tumor antigen peptides of the present invention "work" such that the requirements of 35 U.S.C. § 112, first paragraph are met.

Based upon the above, it is clear that subject matter recited in the claims is enabled by the specification. The rejection under 35 U.S.C. § 112, first paragraph, is therefore improper and should be withdrawn.

***Conclusion***

The facts regarding the protein and peptides of the present invention (i.e., (1) their activity is confirmed in the instant specification, and (2) they have been studied in clinical trials) show that the present invention meets the requirements of utility under 35 U.S.C. § 101 as well as written description and enablement under 35 U.S.C. § 112, first paragraph. The Examiner is therefore

respectfully requested to withdraw the pending rejections and allow the application.

***Summary***

All of the present claims define patentable subject matter such that this application should be placed into condition for allowance. The Examiner is respectfully requested to issue a Notice of Allowability indicating that claims 6, 7, 9, and 12 are allowed.

Pursuant to the provisions of 37 C.F.R. §§ 1.17 and 1.136(a), the Applicants hereby petition for an extension of two (2) months to January 29, 2004, in which to file a reply to the Office Action. The required fee of $420.00 is enclosed herewith.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees

required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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Attachments:
Exhibit 1 - English translation of H. Yamana and K. Itoh, *Jap. J. Cancer Chemother.*, 27(10):1477-1488 (2000);
Exhibit 2 - Mine et al.,*Biotherapy* 15(3):181 (2001) and English Translation thereof
Exhibit 3 - Slingluff et al., *Clinical Cancer Research*, 7:3012-3024 (2001)
Exhibit 4 - Rosenberg et al., *Nature Medicine*, 4(3):321-327 (1998)
Exhibit 5 - Marchand et al., *Int. J. Cancer*, 80:219-230 (1999)
Exhibit 6 - Renkvist et al., *Cancer Immunol. Immunother.* 50:3-15 (2001)

Vo. 27, No. 10, September 2000


REVIEW

# CANCER VACCINE

Hideaki Yamana i*1, Kyogo Itoh **2

*(Jpn J Cancer Chemother* 27(10): 1477-1488, *September*, 2000)

Specific Immunotherapy with Cancer Vaccines: Hideaki Yamana*1 and Kyogo Itoh*2 (*1*Multidisciplinary* Treatment Center, *and* *2Immunology, Kurume University, School of Medicine)

**SUMMARY**

With the recent progress in molecular biology and gene technology, many new cancer-specific antigens have been identified. Many studies have demonstrated the role of HLA class I-restricted cytotoxic T lymphocytes (CTLs) in cancer specific-immunotherapies. We have also established HLA-A24- and A26- restricted and cancer-specific CTLs from a patient with squamous cell carcinoma of the esophagus. Using CTLs, we identified a new gene SART-1 by cDNA-expression cloning and some SART-1-derived cancer rejection peptides were also identified. Further more, using the same approach, we identified a *cyclophilin* B gene that encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-specific CTLs. Now we are performing phase I trials using these peptide vaccines and have found an increase in CTL precursor frequency in some cases in an in vitro study. However, other recent studies have reported that many tumors escape from CTL recognition by downregulation of HLA class I expression. Moreover, most cancer cells produce a suppressor agents against the immune system. Therefore, we must resolve these major problems to produce successful cancer vaccine therapy soon. **Key words**: Cancer-specific immunotherapy, Cancer antigen, Cancer vaccine, Address request for reprints to: Dr. Hideaki Yamana, Multidisciplinary Treatment Center, Kurume University, School of Medicine, 67 Asahi-machi, Kurume 330-0011, Japan

## INTRODUCTION

With the recent progress in basic immunology, the presence of tumor-specific antigens recognized by cytotoxic T cells were proved scientifically from melanoma in 1991, and the conception of the presence of the cancer rejection antigens and the immunologically eliminating function has been establish d. In this way, the presence of tumor-specific antigens rejected by immunocompetent cells was elucidated also in human malignant tumors as well as shown in animal experiment leading to a new phase in

cancer-specific immunotherapy. With this as a turning point, studies on the analysis of cancer antigens have actively been conducted in many facilities. As a result, various antigens specific for human malignant tumors have been identified, and clinical tests for cancer vaccine therapy that employs cancer-specific antigens have been attempted in order to treat malignant tumors.

In the present paper, the present situation of cancer vaccine will generally be stated while focusing on the cancer peptide vaccine that we have developed, and problems at present and future prospects will be studied.

## I. TUMOR-SPECIFIC ANTIGENS RECOGNIZED BY CANCER-SPECIFIC CYTOTOXIC T CELLS (CTLs)

It was known in animal experiment that CTLs kill cancer cells in a major histocompatibility antigen (MHC)-restricted and antigen-specific manner. However, it was only a dozen years ago that the mechanism of recognition of antigen molecules by the CTL has been elucidated, and it was found out that about 8 to 15 amino acids are bound to a small groove constructed by MHC class I or class II domain to become an antigen molecule thereby being recognized by T cells. On the basis of the development of the basic immunoscience, Boon et al. [1] identified MAGE gene encoding an antigen molecule, which is presented on an HLA class I molecule of a human melanoma cell, and which becomes a target of CTLs. Although there are several ways of searching for a cancer rejection antigen recognized by CTLs, cDNA-expression cloning developed by Boon et al. has most successfully isolated novel antigens. Recently, novel antigen molecules have been identified also by SEREX[2] that is a simple expression cloning method that employs antibodies in serum of a cancer patient. Major human cancer rejection antigens, HLA class I restriction and peptides thereof are shown in Table 1

Table 1 Types of human cancer rejection antigens and HLA class I restriction and peptides thereof

| Cancer rejection antigen | HLA restriction | Peptide |
|---|---|---|
| 1. Cancer-testis (CT) antigen | | |
| MAGE-1 | A1 | EADPTGHSY |
| | Cw 16 | SAYEPRKL |
| MAGE-3 | B44 | MEVDPIGHLY |
| | A1 | EVDPIGHLY |
| BAGE | A2 | FLWGPRALV |
| GAGE-1, GAGE-2 | Cw16 | AARAVFLAL |
| RAGE | CW6 | YRPRPRRY |
| NA 17-A | B7 | SPSSNRIRNT |
| NY-ESO-1 | A2 | VLPDVFIRC |
| | A2 | QLSLLMWIT |
| | A2 | SLLMWITQC |
| | A2 | SLLMWITQCFL |
| 2. Differentiation antigen | | |
| MART-1 | A2 | AAGIGILTV |
| | A2 | EAAGIGILTV |
| | A2 | ILTVILGVL |
| | B45 | AEEAAGIGIL |
| | B45 | AEEAAGIGILT |
| gp 100/Pmel 17 | A2 | KTWGQYWQV |
| | A2 | AMLGTHTMEV |
| | A2 | MLGTHTMEV |
| | A2 | ITDQVPFSV |
| | A2 | YLEPGPVTA |
| | A2 | LLDGTATLRL |
| | A2 | VLYRYGSFSV |
| | A2 | SLADTNSLAV |
| | A3 | ALLAVGATK |
| | A24 | VYFFLPDHL |
| tyrosinase | A1 | DAEKCDICTDEY |
| | A2 | MLLAVLYCL |
| | A2 | YMNGTMSQV |
| | A24 | AFLPWHRLF |
| | B44 | SEIWRDIDF |
| | DR4 | QNILLSNAPLGPQFP |
| | DR4 | SYLQDSDPDSFQD |
| TRP2 | A31 | LLPGGRPYR |
| | A33 | LLPGGRPYR |
| PSA-1 | A2 | FLTPKKLQCV |
| PSA-3 | A2 | VISNDVCAQV |
| PSA-9 | A3 | QVHPQKVTK |
| PAP-1 | A2 | LLLARAASL |
| PAP-5 | A2 | ALDVYNGLL |
| PAP-7 | A2 | VLAKELKFV |
| cdr 2-1 | A2 | KLVPDSLYV |
| cdr 2-2 | A2 | SLLEEMFLT |

| | | |
|---|---|---|
| 3. Cancer-specific variant antigen | | |
| variant CDK 4 | A2 | ACDPHSGHFV |
| variant β-catenin | A24 | SYLDSGIHF |
| LB33 (MUC-1) | B44 | EEKLIVVLF |
| variant gp 75 (TRP-1) | A31 | MSLQRQFLR |
| variant HLA-A2 | A2 | CVEWLRIYLENGK |
| CASP-8 | B35 | FPSDSWCYF |
| 4. Antigen highly expressed in cancer | | |
| p15 | A24 | AYGLDFYIL |
| HER 2/neu | A2 | IISAVVGIL |
| PRAME | A24 | LYVDSLFFL |
| GnT-V | A2 | VLPDVFIRC |
| CEA | A2 | |
| Muc-1 | A11 | STAPPHGV |
| 5. Rejection antigens for squamous cell carcinoma | | |
| SART-1 | A24 | EYRGFTQDF |
| | A26 | KGSGKMKTE |
| | A26 | KLDFFALLK |
| | A26 | VLSGSGKSM |
| SART-2 | A24 | DYSARWNEI |
| | A24 | SYTRLFLIL |
| SART-3 | A24 | VYDYNCHVDL |
| | A24 | AYIDFEMKI |

**1. ANTIGENS EXPRESSED IN VARIOUS CANCERS AND NORMAL TESTIS**

Examples of cancer-testis antigens, which are not expressed in normal tissue except for the testis, but are widely expressed in various cancers, include MAGE-1, MAGE-3, BAGE, GAGE-2, RAGE, NA 17-A, NY-ESO-1 and the like[3-8].

**2. DIFFERENTIATION ANTIGENS**

1) Melanosome proteins

Melanosome proteins are differentiation antigens that are expressed in common in normal melanocytes and melanoma. Examples thereof include MART-1/Melan-A, gp 100/Pmel 17, tyrosinase, TRP 2 [9-14] and the like. Many of the identified peptides are HLA-A2-restricted peptides, and are also expressed in melanocytes that are normal cells. Therefore, there is presumed the possibility that these peptides are target molecules in an autoimmune disease in which melanocytes are destroyed.

**3. OTHER ANTIGEN MOLECULES**

Among differentiation antigens other than the melanosome

proteins, examples of well-known molecules that are expressed on the surface of cancer cells include prostate-specific membrane antigen (PSMA) and prostate-acid phosphate (PAP)[15-17], which are expressed in prostate cancer. On the other hand, cdr2 antigen expressed in the brain was identified from a female patient with a tumor of a genital organ[18], which causes paraneoplastic cerebellar degeneration (PCD). These antigens are expected to elucidate disease states of malignant tumors accompanied by PCD and to develop a new therapy etc.

**4. VARIANT ANTIGENS SPECIFIC FOR TUMORS**

Variant antigens specific for tumors are antigens peculiar to cancer, which are produced by gene alteration that occurs in the process of cancerization, and variant CDK4 antigen, variant β-catenin antigen, MUM-1 antigen, CASP-8 and the like are known[19-23]. An example of the case where an intron is translated includes variant gp 75. Although these antigens are specific for cancer, gene alterations are different among the individual tumors. Thus, they are unlikely to serve as vaccine molecules for wide clinical application.

**5. ANTIGENS HIGHLY FREQUENTLY EXPRESSED IN CANCER**

There is a case in which antigens ubiquitously expressed as non-variants in many normal cells and cancer cells are sometimes recognition molecules for the CTL, and p15, HER 2/neu, CEA, MUC-1 and the like are well known[24-27].

**6. REJECTION ANTIGENS FOR SQUAMOUS CELL CARCINOMA**

We have reported that cancer antigen peptides recognized by HLA-A2602-restricted CTLs induced from a patient with squamous cell carcinoma of the esophagus are present[28]. Furthermore, we have discovered that HLA-A24- and HLA-A2-bound cancer antigen peptides are present not only in squamous cell carcinoma of the esophagus or lung, but also in adenocarcinoma of the lung, ventriculus, large intestine and mammary gland[29-32], and have proven that a cancer regression antigen is present in cancers other than melanoma.

## II. THE PRESENT SITUATION OF CANCER VACCINE IN THE UNITES STATES AND EUROPE AND RESULTS THEREOF

**1. MELANOMA**

As a pilot study for 12 patients with HLA-A1-positive metastatic melanoma, single administration of MAGE-3 peptide was conducted on them at Ludwig Cancer Research Institute in 1995[33]. The results thereof revealed that the treatment has been completed in 6 cases and that, among the 6 cases, partial response (PR) was recognized in 3 cases and the rest of the 3 cases were progressive disease (PD). It was als reported in

1999 that, a tumor regression effect was obtained in 7 out of 25 cases in which the treatment has been completed[34]. In 1998, Rosenberg et al. of the National Cancer Institute (NIH) conducted administration of a gp 100 derived peptide in combination with incomplete Freund's adjuvant (IFA) as Phase I Test for patients with HLA-A2 positive metastatic melanoma, and HLA-A2-restricted and melanoma-specific CTLs were induced in peripheral blood mononuclear cells (PBMC) of 20 cases out of 21 cases, and the effect of the peptide vaccine was confirmed for the first time in an *in vivo* clinical test. Furthermore, it is reported that, when high-dose IL-2 was used as an adjuvant, a tumor regression effect was recognized in 13 cases out of 31 cases[35]. However, it was also reported that the distinct tumor regression effect was recognized in 16 % of the samples even in the case of single administration of high-dose IL-2[36]. Thus, further study is required from now on in order to judge the clinical effect of the peptide vaccine on melanoma. Furthermore, in 1998 in Switzerland, there was reported the results of Phase I Test in cell therapy wherein several peptides identified in melanoma were sensitized *ex vivo* to dendritic cells (DC) collected from a patient, and the sensitized product was transferred to the interior of the body[37]. Out of 16 cases of metastatic melanoma, complete response (CR) was recognized in two cases, while partial response (PR) was recognized in 3 cases.

**2. COLON CANCER**

As an adjuvant postoperative therapy for Stage II, III colon cancer patients, phase III test for vaccine therapy using autologous cancer cells irradiated with radioactive rays and BCG was conducted by Eastern Cooperative Oncology Group (ECOG). It was reported that, in a comparison of prognosis between a group of patients who underwent surgical operation alone and a group of patients who underwent the surgical operation and the vaccine therapy, the vaccine therapy group showed a better tendency, i.e., $p=0.078$ in a disease free survival curve, while $p=0.13$ in a total survival curve. Besides the above, some clinical tests using vaccine antibodies associated with tumor antigens have been conducted, but as for their efficacy, no clear conclusion has been obtained yet.

**3. PROSTATE CANCER**

The vaccine therapy for prostate cancer mainly includes a combination of a cell therapy using DCs, to which HLA-class I-restricted and PSMA-specific peptide has been sensitized, and GM-CSF, or a vaccine therapy comprising transferring GM-CSF gene into autologous tumor cells, irradiating the cells with radioactive rays. Howev r, their clinical ff ct has not reached a standard constant level yet[39-42].

**4. RENAL CELL CANCER**

The vaccine therapy for renal cell cancer mainly includes a cytokine such as IL-2, interferon (IFN)-α, β, γ and GM-CSF, and an autologous cancer cell vaccine, or a DC therapy[43-47]. Studying the results of clinical tests conducted this time in Germany, it was reported that a hybrid vaccine composed of autologous cancer cells and DCs has achieved antitumor effects of CR/PR (complete remission/partial remission), and thus future achievement is expected.

### 5. OTHERS

Some vaccine therapies have been performed[49^51] also on bladder cancer, lung cancer and uterine cancer, but no clear conclusion regarding their clinical effects has been obtained yet.

## III. PRESENT SITUATION OF CANCER VACCINE IN JAPAN

Also in Japan, in the late 1990's, clinical tests using MAGE-3 peptide, CEA vaccine[52], MUC-1 peptide and the like were performed, and a part of them has been disclosed at a seminar, a society and so on. Further, using HER2/neu peptide, Shuku et al. have started clinical tests on HLA-A24-positive adenocarcinoma patients.

For the purpose of identifying rejection antigens for carcinoma and achieving their clinical application, so far, we have identified seven rejection antigens and a dozen peptide antigens encoded thereby. SART-1[53-59], SART-2[60], SART-3[61] and SART-4 were identified in cDNA of squamous cell carcinoma of the esophagus, while cyclophilin B[62] (CypB), ART-1 and ART-4 were identified in cDNA of bladder cell adenocarcinoma. Further, we have also found that HLA-A24-restricted and cancer-specific CTLs recognize $p56^{lck}$ (Lck) [63], and synthesized 9-mer and 10 mer Lck peptides having binding motifs for HLA-A24. Among these peptides, phase I test for SART-1 peptide, SART-3 peptide, CypB peptide and Lck peptide was performed on HLA-A24- or HLA-A26-positive squamous cell carcinoma of the esophagus and the lung, HLA-A24-positive colon cancer and breast cancer, HLA-A24-positive lung cancer, and HLA-A24-positive and Lck-positive metastatic carcinoma, respectively, or the above peptides are being in readiness for the test.

The summary of SART-1, SART-3 and CypB on which clinical tests have already been conducted and CTL-inducing capacity of each peptide will be introduced.

Incidentally, these cancer antigen peptides that induce CTLs are shown in Fig. 2.

Table 2 CANCER ANTIGEN PEPTIDES ON WHICH CLINICAL TESTS ARE BEING PERFORMED AT KURUME UNIVERSITY HOSPITAL

| Antigen | HLA-A locus | Peptide |
|---|---|---|
| SART-1 | HLA-A26 (01, 02, 03) | KGSGKMKTE |
| | HLA-A2402 | EYRGFTQDF |
| SART-3 | HLA-A2402 | VYDYNCHVDL |
| | | AYIDFEMKI |
| CypB | HLA-A2402 | KFHRVIKDF |
| | | KYHRVIKDF (modified peptide) |
| | | DFMIQGGDF |
| | | DYMIQGGDF (modified peptide) |

**1. SART-1**

1) *SART-1* gene[53]

CTLs (KE 4-CTLs) were induced using an autologous cancer cell line (KE4) as an antigen from PBMC of a patient with squamous cell carcinoma of the esophagus (HLA-A2401/A2601). A gene encoded by the KE-4-CTL was obtained by a cDNA-expression cloning method using the KE-4-CTL. Using the gene as a probe, a presumably almost full-length SART-1 gene (2,506 bp) was identified from the KE4 cell line and a cDNA library of healthy human PBMC.

*2) SART-1 antigen*

*SART-1* mRNA was expressed in almost all the healthy tissues, but the KE 4-CTL was not able to recognize autologous non-cancer cells. The expression at an actual protein level was analyzed by the Western blot method with a polyclonal antibody that was prepared using SART-1 fusion protein as an antigen. Expression of 125-kDa SART-1 protein was recognized not only in all the cancer cell lines that were searched but also in a nuclear fraction of a normal cell line having growth activity. However, the expression was not detected in healthy tissues other than the testis and fetal liver (Table 3).

On the other hand, it was considered that the firstly cloned 990-bp gene codes for another protein consisting of 259 amino acids. Then, a polyclonal antibody was prepared. The polyclonal antibody recognized a 43 kDa protein in a cytoplasmic fraction, and, in the case of human squamous cell carcinoma cell lines, the expression of the protein was recognized in most of head and neck cancer, squamous cell carcinoma of the esophagus and squamous cell carcinoma of the lung, and the expression thereof was also recognized in many tissues of squamous cell carcinoma. However, the expression of the protein was not recognized in outgrowing normal cells and healthy human tissues other than the testis and fetal liver, so that the 43-kDa cytoplasmic protein was presumed to be a cancer rejection antigen.

3) *SART-1* peptide

a. HLA-A26-binding SART-1 peptide

Twenty two oligopeptides corresponding to binding motifs for HLA-A-2601 molecule in SART-1 molecule were synthesized. VA13 cells in which HLA-A2601 gene has been introduced were sensitized with these peptides and then the recognition by KE 4-CTLs was measured by the IFN-γ production assay and $^{51}Cr$ release assay. As a result, IFN-γ production was significantly induced in 3 peptides each consisting of 10 amino acids (10-mer), and, as for the cytotoxic activity, SART-$1_{736-745}$ showed a highest value. Further, it was found that each moiety of 9-mer peptide (KGSGKMKTE) of the 10-mer peptides was recognized by KE 4-CTLs and specific CTL induction activity against the KE 4-cell line was studied. As a result, only PBMC induced by SART-$1_{736-744}$ peptide and its sublines showed specific and high cytotoxic activity. Thus, they were judged to be clinically applicable and clinical tests were performed (Fig. 1).

b. HLA-A24-binding SART-1 peptide

The KE 4-CTL has another HLA class I allele A2402. In the same manner as in the HLA-A2601-restricted CTL cell line, an HLA-A2402-restricted CTL cell line was established. The analysis using this CTL cell line revealed that a peptide antigen[5] that strongly binds to HLA-A2402 was present in a region consisting of 62 amino acids from the 3' terminus, which is common to both of the 125 kDa- and 43 kDa-proteins. Then, the peptides recognized by the KE 4-CTL cell line were studied using 12 different SART-1 protein-derived peptides corresponding to HLA-A2402-binding motifs. As a result, the original peptide of SART-$1_{690-698}$ (EYRGFTQDF) was recognized most strongly. It was found that when sensitized with the peptide, HLA-A24-restricted cancer-specific CTLs were induced not only from PBMC of a cancer patient but also from PBMC of a healthy human, suggesting the possibility of clinical application thereof. Therefore, clinical tests were performed (Fig. 1).

## IV. PROBLEMS OF CANCER VACCINE THERAPY AND FUTURE PROSPECTS

The cancer vaccine therapy at present is still in trial stages and is yet to reach a level of discussing its clear efficacy. However, it is difficult to expect a remarkable tumor regression effect from the results of the above clinical tests, and thus further study is required. The problem of the cancer peptide vaccine therapy at present is that, even if CTL precursor cells increase, the cells have a weak cytotoxic effect. Thus, it is required to study how the precursor cells that have outgrown by the induction are activated.

Furthermore, even if CTLs increase in a living body, there is a limit in their number. Therefore, destroying carcinoma only by CTLs is considered to be impossible. In order to achieve a clear antitumor effect in imaging diagnostic, it is required to use all the immunocytes having antitumor activity such as NK cells and LAK cells, and the corelationship with DCs, CD 4 positive cells and the like becomes important. Furthermore, a mechanism of escaping from immunity that a tumor itself has and production of immunosuppressing products by the tumor and the like must be elucidated by further study. We believe that a shortcut for establishing the vaccine therapy is to solve the above problems step by step from both aspects of the basic study and the clinical study without seeking only the clinical effect of the cancer vaccine therapy.

## CONCLUSION

Ten years have passed since the cancer rejection antigen MAGE-1 was identified, however, the therapy targeting human cancer rejection antigens is in stages of clinical tests. Thus, on the basis of the results obtained, study must further be carefully conducted in future. We consider that the most effective method as the cancer-specific immunotherapy at present is the adoptive therapy comprising activating a patient's lymphocytes in the presence of IL-2 using autologous cancer cells as antigens, and injecting them directly into the foci. However, this treatment is specific for individual patients. Considering the present situation in which about 8 million or more cancer patients are present, this treatment can never be judged to be an effective treatment. In order to perform effective cancer-specific treatment on such a great number of patients, it is required by all means to develop cancer vaccines with high efficacy and prove their efficacy in clinical tests.

Cited literatures:

Table 3 EXPRESSION OF SART PROTEINS IN NORMAL AND TUMOR CELLS AND TISSUES (BY WESTERN BLOT ANALYSIS)

| | | SART-1 | | | | SART-2 | | SART-3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | cell line | | tissue | | cell line | tissue | cell line | | tissue | |
| | | cytoplasm | nuclei | cytoplasm | nuclei | | | cytoplasm | nuclei | cytoplasm | nuclei |
| Normal cells and tissues | PBMC | 0/5 | 0/5 | | | 0/3 | | 0/5 | 0/5 | | |
| | blast-transformed PBMC | 0/2 | 2/2 | | | | | 3/3 | 0/3 | | |
| | fibroblast | 0/2 | 2/2 | | | 0/1 | | 2/2 | 0/2 | | |
| | fetal liver | | | 1/1 | 1/1 | | 0/1 | | | 1/1 | 0/1 |
| | neonatal liver | | | 0/1 | 0/1 | | 0/1 | | | 0/1 | 0/1 |
| | liver | | | 0/1 | 0/1 | | 0/1 | | | 0/10 | 0/10 |
| | testis | | | 3/3 | 1/1 | | 0/2 | | | 2/2 | 2/2 |
| | placenta | | | 0/2 | 0/1 | | 0/1 | | | 1/1 | 0/1 |
| | esophagus | | | 0/4 | 0/2 | | 0/1 | | | 0/3 | 0/3 |
| | pancreas | | | 0/1 | 0/1 | | 0/1 | | | | |
| | lung | | | | | | 0/1 | | | | |
| | kidney | | | | | | 0/1 | | | | |
| | thymus | | | | | | 0/1 | | | | |
| | ovary | | | | | | 0/1 | | | | |
| Cancer cells and tissues | head and neck squamous cell carcinoma | 3/5 | 2/2 | 7/7 | 2/2 | 8/8 | 15/16 | 2/2 | 2/2 | 14/20 | 10/20 |
| | esophageal squamous cell carcinoma | 4/6 | 5/5 | 18/30 | 3/5 | 5/5 | 5/9 | 8/8 | 8/8 | 3/5 | 4/5 |
| | lung squamous cell carcinoma | 3/3 | 2/2 | 8/17 | 3/4 | 3/6 | 4/14 | 4/4 | 4/4 | 7/10 | 6/10 |
| | lung adenocarcinoma | 3/6 | 3/3 | 16/35 | 7/7 | 2/2 | 6/10 | 3/3 | 3/3 | 5/8 | 4/8 |
| | breast cancer | | | | | 0/3 | 0/16 | 12/12 | 12/12 | 4/4 | 4/4 |
| | melanoma | 0/2 | 1/1 | 0/10 | | 1/1 | 3/8 | 2/2 | 2/2 | 8/9 | 8/9 |
| | leukemia | 0/16 | 4/4 | 0/10 | 4/4 | 15/15 | 3/4 | | | | |

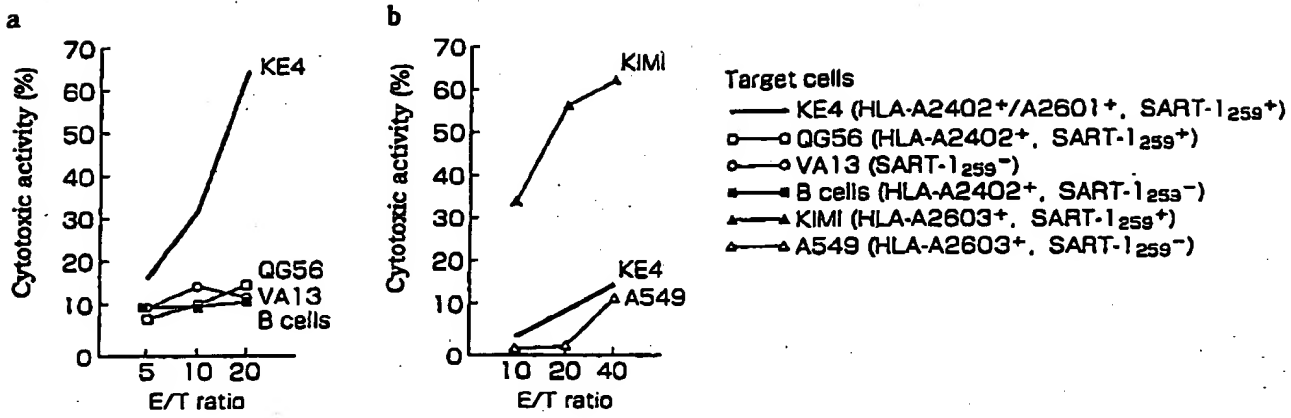


Fig. 1 Cytotoxic activity of HLA class I-restricted and cancer-specific CTLs induced with SART-1 peptide from peripheral blood lymphocytes (based on $^{51}Cr$ release assay)

a: HLA-A24-restricted SART-$1_{690-698}$ b: HLA-A26-restricted SART $1_{736-744}$

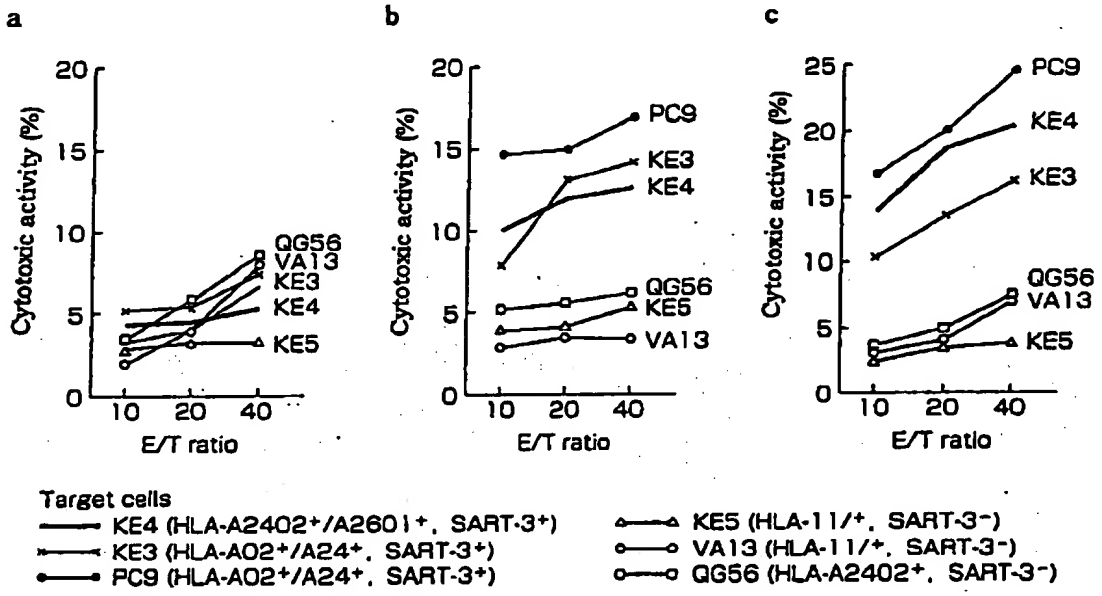


Fig. 2 Cytotoxic activity of HLA-A24-restricted and cancer-specific CTLs induced with SART-3 peptide from peripheral blood lymphocytes (based on $^{51}Cr$ release assay)
a: no stimulation with peptides b: SART-$3_{109-118}$ c: SART-$3_{315-323}$

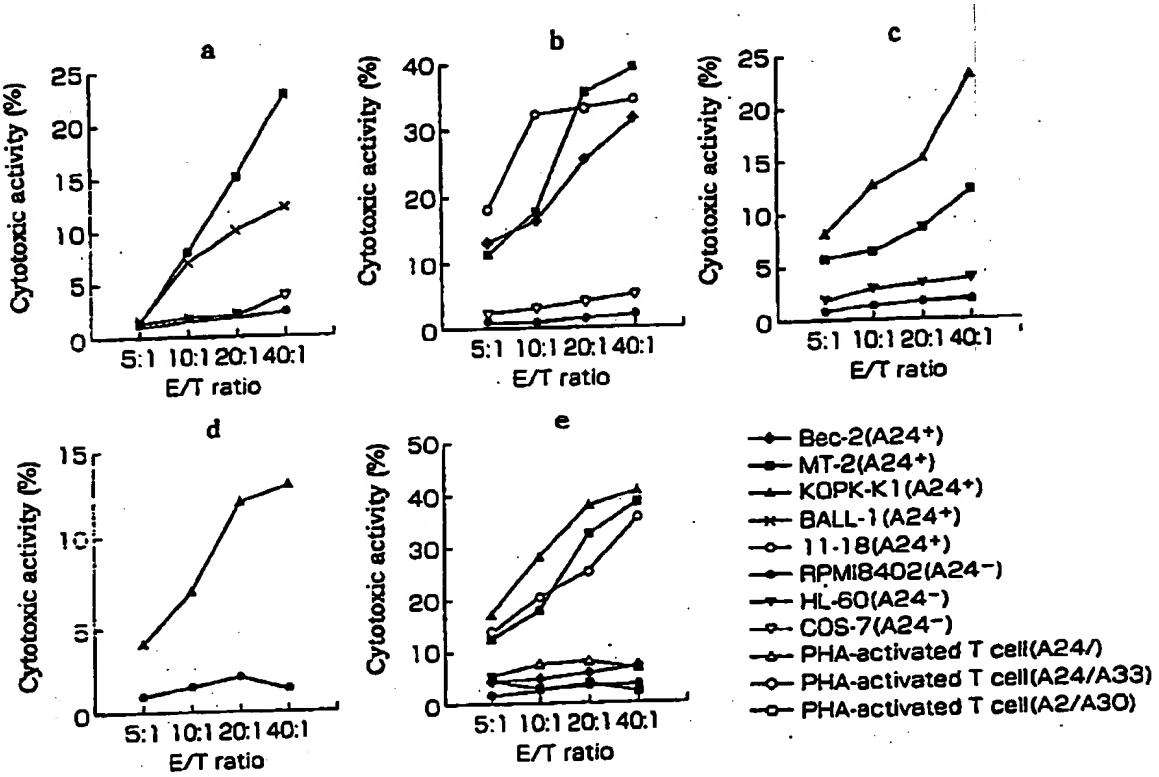


**Fig. 3** Cytotoxic activity of HLA-A24-restricted and cancer-specific CTLs induced with CypB peptide from peripheral blood lymphocytes (based on $^{51}Cr$ release assay)

a: CTL activity of the leukemia patient stimulated three times with $CypB_{84\text{-}92}$
b: CTL activity of the leukemia patient stimulated three times with $CypB_{84\text{-}92}$ modified peptide
c: CTL activity of the gastric cancer patient stimulated three times with $CypB_{84\text{-}92}$ modified peptide
d: CTL activity of the healthy human stimulated three times with $CypB_{84\text{-}92}$ modified peptide
e: CTL activity of the healthy human stimulated three times with $CypB_{91\text{-}97}$ modified peptide


Vo. 15, No. 3, May 2001

The 13th Assembly of Academic Conference of Japan Society of Biotherapy
Symposium Fundamental and Practice of Cancer Vaccine Therapy

# Interim Analysis of Cancer Peptide Vaccine Phase I Test

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[Purpose] We are carrying out six clinical test for the purpose of developing vaccine therapy of highly advanced esophageal cancer, lung cancer, colon cancer, breast cancer, gynaecological cancer, metastatic cancer using HLA-A24-restricted peptides. We will report the interim analysis of phase I clinical test for peptide vaccine wherein SART-1, SART-3 and cyclophilin B (Cyp B) are tested.

[Method] The phase I clinical test was started in May, 1999, wherein SART1 peptide vaccine was used in combination with IFA. Clinical tests for Cyp B and SART3 peptides were then started. The subjects of the phase I clinical test were esophageal/lung squamous cancer patients (SART1), colon/breast cancer patients (SART3) and lung cancer patients (Cyp B). The primary and secondary end points of each test are the evaluation of safety and the change of immunoreactivity, respectively. The dosing plan of peptide consisted of three stages in total, i.e., initially, 0.3 mg/body in all the tests, then increased to 1.0 mg/body and 3.0 mg/body. Every cohort was consisted of 3 to 4 cases. In the clinical tests now in progress, SART1 is tested at 1.0 mg/body, SART-3 is tested at 3.0 mg/body, and Cyp B is tested using a variant peptide.

[Results]

1) The adverse events resulted from peptide vaccine observed to date are only reddening, swelling and slight pain at the site of injection.

2) In the pre-administration intracutaneous reaction test, positive reaction for type I allergy was observed in 3/3 cases regarding $CypB_{84-89}$ (KFHRVIKD) and 3/5 cases regarding $SART\text{-}3_{315-324}$ (AYIDFEMKI). These two peptides were withdrawn from the test.

3) Induction or enhancement of HLA class I-restricted tumor-specific CTLs due to administration of peptides was observed in 10/11 cases. The number of CTL precursor cells after administration of peptide was 1/159 - 1/1,564 (mean, 1/347).

4) The number of CTL precursor cells to administered peptide was 1/635 - 1/1,246 (mean, 1/1,688).

5) CTL-associated effects were observed in some cases due to administration of peptides.

6) The number of CTL precursor cells and reactive peptide were revealed to vary depending on the case.

[Discussion]

The above-mentioned results indicate that it is necessary to consider the following matters in the future research.

1) Phase I test using certain peptides should be started, which peptides are positive in a pretest conducted before administration wherein the presence or absence of CTL precursor cells for 11 kinds of peptides is examined.

2) Phase I/phase II test using an effective adjuvant such as IL-2 or the like for the purpose of clinical effects should be started.

3) Immunological analysis on type I allergy resulted from intracutaneous reaction.

特集

第13回 日本バイオセラピィ学会学術集会総会
シンポジウム 癌ワクチン療法の基礎と臨床

# 癌ペプチドワクチン第Ⅰ相試験の中間解析

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【目的】癌拒絶抗原ペプチドを用いたワクチン療法の開発を目指して，現在HLA-A24拘束性のペプチドについて六つの臨床試験を高度進行食道癌，肺癌，大腸癌，乳癌，婦人科癌，転移癌に対して実施中である。今回はSART1，SART3，およびcyclophilin B（Cyp B）のペプチドワクチン第Ⅰ相試験の中間解析結果について報告する。

【方法】1999年5月よりSART1ペプチドワクチンとIFA併用による第Ⅰ相試験を開始し，続いてCyp BとSART3ペプチドワクチンの臨床試験を開始した。SART1は食道/肺扁平上皮癌患者を，SART3は大腸/乳癌患者を，またCyp Bは肺癌患者を対象として第Ⅰ相試験を開始した。いずれの試験も，primary endpointは薬剤の安全性の評価であり，secondary endpointは免疫反応性の変化である。ペプチドの初回投与量はいずれの試験においても0.3 mg/bodyに設定し，その後の増量計画は1.0 mg/bodyと3.0 mg/bodyの計3段階とし，各コホートの症例数は3～4例である。現在，SART1は1.0 mg/bodyの試験を，SART3は3.0 mg/bodyを，Cyp Bは改変体ペプチドによる試験を続行中である。

【結果】現在までのペプチドワクチンによる有害事象は，以下のとおりである。1）薬剤注入部局所の発赤・腫脹，軽度の痛みのみである。2）薬剤投与前の皮内反応試験で，Cyp $B_{84-89}$（KFHRVIKD）に対して3例中3例が，また$SART3_{315-323}$（AYIDFEMKI）に対して5例中3例がⅠ型アレルギー陽性となり，これらペプチドの投与が中止された。3）ペプチドによるHLA class Ⅰ拘束性癌特異的CTLの誘導もしくは増強が11例中10例に認められた。ペプチド投与後のCTL前駆細胞数は1/159～1/1,564（mean, 1/347）であった。4）投与ペプチドに対するCTL前駆細胞数は，1/635～1/5,246（mean, 1/1,688）であった。5）ペプチド投与によって，数例にCTL随伴効果が認められた。6）症例ごとにCTL前駆細胞数および反応するペプチドが異なることが判明した。

【考察】上記成績から，今後の研究として以下の項目について検討する必要性がある。1）11種類のペプチドに対するCTL前駆細胞数の有無を，ペプチド投与前にチェックし，陽性ペプチドのみを投与する第Ⅰ相試験の開始。2）IL-2などの有効なアジュバントを併用することにより，臨床効果を目的とした第Ⅰ/Ⅱ相試験の開始。3）皮内反応により出現したⅠ型アレルギー対する免疫学的解析。




# Phase I Trial of a Melanoma Vaccine with $gp100_{280-288}$ Peptide and Tetanus Helper Peptide in Adjuvant: Immunologic and Clinical Outcomes[1]

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## ABSTRACT

**A melanoma vaccine composed of HLA-A2-restricted peptide YLEPGPVTA ($gp100_{2M0}$), with or without a modified T-helper epitope from tetanus toxoid AQYIKANSKFIGITEL, has been evaluated in a Phase I trial to assess safety and immunological response. The vaccines were administered s.c. in either of two adjuvants, Montanide ISA-51 or QS-21, to 22 patients with high-risk resected melanoma (stage IIB–IV). Local and systemic toxicities were mild and transient. We detected CTL responses to the $gp100_{2M0}$ peptide in peripheral blood in 14% of patients. Helper T-cell responses to the tetanus helper peptide were detected in 79% of patients and had a Th1 cytokine profile. One patient with a CTL response to gp100 had a recurrence in a lymph node 2 years later; her nodes contained $CD8^+$ cells reactive to $gp100_{2M0}$ (0.24%), which proliferated in response to peptide. The overall survival of patients is 75% (95% confidence interval, 57–94%) at 4.7 years follow-up, which compares favorably with expected survival. Four of 14 patients who completed at least six vaccines subsequently developed metastases, all of which were solitary and surgically resectable. They remain alive and clinically free of disease at last follow-up. Data from this trial demonstrate immunogenicity of the $gp100_{2M0}$ peptide and suggest that immune responses may persist long-term in some patients. The frequency and magnitude of the CTL response may be improved with more aggressive vaccination regimens. Although this Phase I study was not intended to evaluate clinical benefit, the excellent survival of patients on this protocol suggests the possibility of a benefit that should be assessed in future studies.**

## INTRODUCTION

Human melanoma cells express antigens recognized by CTLs. These include antigens created by unique, random mutations and those that are commonly expressed on melanomas from many different patients (1). The most widely shared melanoma antigens recognized by CTLs are derived from melanocyte differentiation proteins including tyrosinase, gp100, MART-1/Melan-A, and gp75. Over half a dozen peptides originating from gp100 have been defined in the HLA-A2 setting (2–5), and at least 4 of them are confirmed to be naturally processed and presented in melanoma (4).

The nonamer peptide YLEPGPVTA was defined as an epitope for CTLs from five different HLA-A2 patients with melanoma (2). This sequence represents residues 280–288 of the melanoma differentiation protein gp100 ($gp100_{2M0}$). It is unique in that it could be recognized by CTLs at very low concentrations, at or below the pM level (2). This peptide induces CTL responses *in vitro* in a substantial proportion of patients (6); thus, its immunogenicity has been suggested indirectly. When originally defined, it was identified among peptides eluted from HLA-A2.1 molecules from human melanoma cells when those naturally processed peptides were evaluated by mass spectrometry (2). Thus, $gp100_{2M0\ 2M}$ is naturally processed and presented in this form. It appears to be presented at the cell surface at low copy number in HLA-A*0201$^+$ individuals; yet even at that low copy number its expression is adequate for CTL recognition and tumor cell lysis (2). Because this peptide is recognized by CTLs at low concentration in a significant number of melanoma patients in multiple studies, it is an appealing antigen to evaluate in a clinical tumor vaccine trial.

Vaccines that simultaneously induce helper T cells and CTLs may be more effective than those that induce CTLs only (7, 8). At the time this trial protocol was prepared, however, no class II MHC-restricted epitopes for helper T cells had been

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defined from melanoma proteins. Instead, a tetanus peptide was included in the trial as a nonspecific helper epitope, because virtually all Americans have been vaccinated against tetanus. The peptide QYIKANSKFIGITEL (p2), representing amino acid residues 830–844, has been shown to bind class II MHC molecules of all patients tested and specifically to HLA-DR1, DRw15 (2), DRw18 (3), DR4Dw4, DRw11 (5), DRw13(w6), DR7, DRw8, DR9, DRw52a, and DRw52b, which account for 80–90% of the population (9–11). The $NH_2$-terminal glutamine (Q) residue is highly susceptible to conversion to pyroglutamate. To stabilize the structure, we modified that peptide by addition of an $NH_2$-terminal alanine residue, and this modified tetanus peptide AQYIKANSKFIGITEL ($Tet_{AK830}$) was included in the vaccine protocol. The ability of $Tet_{AK830}$ to induce T-helper responses is not abrogated by addition of one or several residues to the $NH_2$ terminus of the peptide (9–11). The patients were divided into three groups, who were vaccinated with: (*a*) the gp100 peptide alone; (*b*) the gp100 peptide plus the tetanus peptide; or (*c*) a fusion peptide incorporating both peptide sequences.

The optimal adjuvants for vaccinating with peptides had not been well-studied in humans. Thus, we used two different adjuvants for the vaccines in the trial. Montanide ISA-51 (Seppic, Inc.) is a mineral oil-based adjuvant analogous to incomplete Freund's adjuvant, which must be administered as an emulsion. QS-21 (Antigenics; Aquila Biopharmaceuticals, Framingham, MA) is a highly purified, water-soluble saponin that handles as an aqueous solution (12, 13). Both have been used in preclinical studies and in clinical trials and have the ability to induce CTL responses in animals and in humans (10, 14–17).

We have completed a Phase I trial of vaccination with the $gp100_{280\text{–}288}$ peptide YLEPGPVTA, with or without the modified T-helper epitope from tetanus toxoid, AQYIKANSKFIGITEL. Patients were also randomized to either of two different adjuvants Montanide ISA-51 or QS-21. In the present study, we report on safety, immunogenicity, and clinical outcome with this vaccine approach.

## PATIENTS AND METHODS

**Patients.** Patients with resected stage IIB, III, and IV melanoma or patients with minimal metastatic disease were eligible. Entry criteria included ages 18–79, expression of HLA-A2 on peripheral blood mononuclear cells, and expression of gp100 by melanoma cells based on immunohistochemistry. Patients were not eligible if they were pregnant; had received cytotoxic chemotherapy, steroids, IFNs, or other investigational drugs within the preceding 3 months; or if they had ever received a melanoma vaccination. Eligible patients were studied with informed consent and with Institutional Review Board and FDA[4] approval under IND 6453: Phase I Protocol for the Evaluation of the Safety and Immunogenicity of Vaccination

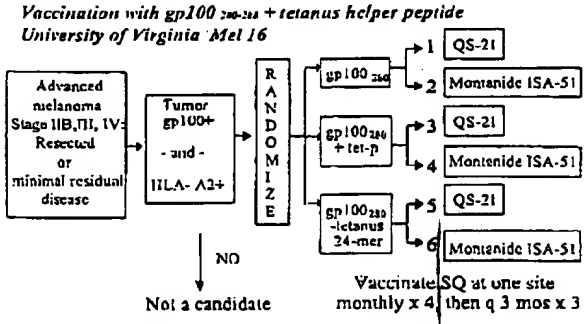


*Fig. 1* Flow diagram for Mel16 peptide vaccine trial. Patients were randomized first to groups 1 and 2, and then subsequent patients were randomized to groups 3–6.

with a Synthetic Melanoma Peptide in Patients with High Risk Melanoma.

**Peptide Vaccine Preparations.** The peptide vaccine preparations used for this trial included peptide $gp100_{280}$ (YLEPGPVTA), tetanus peptide (AQYIKANSKFIGITEL), and fusion peptide gp100-tet (YLEPGPVTAQYIKANSKFIGITEL). Peptides were synthesized at the UVA Biomolecular Core Facility with a free amide $NH_2$ terminus and free acid COOH terminus. Each was provided as a lyophilized peptide, which was then reconstituted in sterile water and diluted with Lactated Ringer's solution (LR, Baxter Healthcare, Deerfield, IL) as a buffer for a final concentration of 67–80% Lactated Ringer's in water. These solutions were then sterile-filtered, placed in borosilicate glass vials, and submitted to a series of quality assurance studies including confirmation of identity, sterility, general safety, and purity, in accordance with FDA guidelines, as defined in IND 6453. Tests of peptide stability demonstrated no decrease in purity or in the peptide concentration, when these peptide solutions were stored at −20°C ~3 years.

QS-21 and Montanide ISA-51 adjuvants were provided by Antigenics (Aquila Biopharmaceuticals) and Seppic, Inc. (Paris, France), respectively, in sterile, single-use vials.

**Immunization Protocol.** Patients received a vaccine containing 100 μg of the HLA-A2-restricted melanoma peptide YLEPGPVTA ($gp100_{280\text{–}288}$), with or without the HLA-DR-restricted tetanus helper peptide AQYIKANSKFIGITEL. The patients were treated in six groups (Fig. 1). Groups 1 and 2 were vaccinated with 100 μg of the gp100 peptide in adjuvant, alone. Groups 3 and 4 were vaccinated with 100 μg of the gp100 peptide plus 190 μg of the tetanus helper peptide. The higher dose of the tetanus peptide was calculated to provide equimolar quantities of the helper and cytotoxic epitopes. Groups 5 and 6 were vaccinated with a 24-mer peptide comprising the amino acid sequences of both the gp100 peptide and the tetanus peptide (YLEPGPVTAQYIKANSKFIGITEL). The first 9 patients were randomized between groups 1 and 2, and the subsequent 13 patients were randomized among groups 3–6. The peptides, in 1-ml aqueous solution, were administered either as a solution/suspension with 100 μg of QS-21 (groups 1, 3, and 5) or as an emulsion with 1 ml of Montanide ISA-51 adjuvant (groups 2, 4, and 6). Patients were immunized at day 0 and months 1, 2, 3, 6,

[4] The abbreviations used are: FDA, Food and Drug Administration; ELISPOT, enzyme-linked immunospot; IL, interleukin; PBL, peripheral blood lymphocyte; DTH, delayed-type hypersensitivity; SI, stimulation index; TCR, T-cell receptor; CI, confidence interval.

9, and 12, with the peptides plus adjuvant, for a total of seven immunizations. With rare exceptions, the vaccinations were administered to the same arm with each vaccine. They were administered s.c. A schematic of the protocol is presented in Fig. 1.

The patients did not receive any concomitant treatments during the course of vaccination. In particular, none of the patients received IFN-α during the course of the vaccinations. IFN was not approved for the adjuvant therapy of melanoma when this study was initiated.

Stopping rules were written such that patients were first entered into groups 1 and 2, with the gp100 peptide plus adjuvant alone, and accrual into groups 3–6 (tetanus peptide added) was begun only after safety was determined for patients in groups 1 and 2. Thus, patients were randomized to groups 1 and 2 initially, and then remaining patients were randomized to groups 3–6. The stopping rule was based on considering the maximum tolerated dose to have been exceeded if two patients of six experienced toxicity of grade 3 or greater. Had that occurred, the trial was to be stopped, with the possibility of considering a revised protocol using a lower dose of antigen or adjuvant.

**Cell Lines Used.** T2 is a mutant human T/B-cell hybrid that lacks the transporter associated with antigen processing (TAP) but expresses HLA-A*0201 (18). It was provided by Peter Cresswell (Yale University, New Haven, CT). HLA typing was performed by microcytotoxicity assay on autologous lymphocytes (One Lambda, Canoga Park, CA).

**Peptides.** Class I MHC-associated peptides used in the laboratory studies included YLEPGPVTA ($gp100_{280\ 288}$; Ref. 2), YMDGTMSQV ($tyrosinase_{368\ 376D}$; Ref. 19), ALLAVGATK ($gp100_{17\ 25}$; Ref. 20), and YLKKIKNSL (Malaria $CSP_{334\ 342}$; Ref. 21), plus the peptides used for vaccination as described above.

**ELISPOT Assays.** Lymphocytes were cultured in complete RPMI 1640 with 10% heat-inactivated human AB serum (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Pen-Strept; Life Technologies, Inc., Grand Island, NY). They were assayed 2 weeks after one or two sensitizations *in vitro* with peptide. For these sensitizations, $2 \times 10^6$ lymphocytes/ml in complete medium were incubated with synthetic peptide (40 μg/ml) for 2 h at 37°C, 5% $CO_2$. The cells were pelleted, resuspended in complete medium containing IL-2 (20 units/ml; Chiron Corp., Emeryville, CA), and cultured in 24-well plates (Linbro; ICN Biomedicals, Inc., Aurora, OH) starting at $3–4 \times 10^6$ cells/well. Complete medium with IL-2 was replaced as needed. When two *in vitro* sensitizations were performed, the second sensitization used autologous irradiated PBLs pulsed with peptide as stimulator cells at a 1:3 stimulator:lymphocyte ratio, which were added to the cultures on day 7. Cells were cultured a total of 14 days prior to evaluation by ELISPOT assay. Nonstimulated lymphocytes were tested in IL-10 ELISPOT assay without prior sensitizations.

Immulon 2 flat-bottomed plates (Dynatech, Chantilly, VA) were coated with anti-IFN-γ monoclonal antibodies (M-700A; Endogen, Woburn, MA) or anti-IL-10 monoclonal antibodies (M-010; Endogen). For IFN-γ assays, lymphocytes were mixed with equal numbers of antigen-presenting cells (T2, C1R-A1, and C1R-A3, depending on HLA type) alone or cells pulsed with peptide (40 μg/ml) in the first row of the plate. Serial dilutions were made, such that responder cell number ranged from 100,000 to 5,000 per well. For IL-10 assays, peptides were added (10 μg/ml) directly to mononuclear cells, without added antigen-presenting cells. Plates were incubated at 37°C, 5% $CO_2$ for 18 h. After extensive washing with 0.025% Tween 20 in water, plates were incubated with a biotin-labeled secondary antibody to IFN-γ (M-701B; Endogen) or IL-10 (M-011-B; Endogen), then washed again, and incubated with avidin conjugated with alkaline phosphatase (13043E; PharMingen, San Diego, CA). After washing, plates were developed with the 5-bromo-4-chloro-3-indolyl phosphate substrate in 1% low melting agarose. The number of blue spots corresponding to the number of cells secreting IFN-γ (or IL-10) were counted in each well, visually, using a Nikon TMS microscope with ×2 objective. Each sample was tested in triplicate at each of several dilutions of lymphocytes. The average number of spots produced by lymphocytes incubated with cells alone was compared with that produced by lymphocytes incubated with cells loaded with peptide. The frequency of T-cells reactive to peptide was calculated based on this difference.

ELISPOT assays were performed on the prevaccine blood sample, and representative samples were taken after each vaccine, ranging from 3 to 7 test dates/patient. Twenty-one of the 22 patients were evaluated in this manner for IFN-γ, with 1 patient excluded because of his very early withdrawal from the study.

**IFN-γ Release Assays.** In selected cases, CTL responses detected by ELISPOT were confirmed in a second assay to measure responses to peptide antigen. T cells sensitized with peptide *in vitro*, as above, were incubated with stimulator cells at a responder:stimulator ratio of 2:1 ($2 \times 10^5$ CTLs and $1 \times 10^5$ target cells/well in a 96-well plate), in assay medium for 24–48 h. IFN-γ released in the medium was quantitated by ELISA using the matching anti-IFN-γ antibody pair M-700A and M-701B (Endogen) in accordance with the manufacturer's directions. Human recombinant IFN-γ (Life Technologies, Inc.) was used for calibration.

**DTH Responses and Recall Antigen Responses.** Patients received injections at separate sites intradermally with each of four standard recall antigens: Trichophyton (Hollister Stier, Spokane, WA); Tetanus (Wyeth-Ayerst Laboratory, Philadelphia, PA); Candida (Hollister Stier); purified protein derivative (PPD, Pasteur Merieux Connaught, Swiftwater, PA), and with 10 μg of the gp100 peptide, without adjuvant, prior to the first vaccine, and the induration at each skin test site was recorded at 24 and 48 h. This was repeated 6 or 12 months after the first vaccine. A positive response is considered an area of induration 10 mm or greater in diameter.

**Proliferation Assay.** PBL samples collected before immunization, after three and after six immunizations, were cultured at $2 \times 10^5$/well in the complete medium with 10% human AB serum: (*a*) without any additional antigen; (*b*) in the presence of bovine albumin (0.5 μg/ml; Sigma Chemical Co., St. Louis, MO); (*c*) with tetanus toxoid (1:2000 dilution of vaccine prep; Wyeth-Ayerst Laboratories); or (*d*) with tetanus helper peptide $Tet_{AN30}$ (10 μM). Cells were plated in triplicate or quadruplicate per condition. On day 5, cells were labeled with

[$^3$H]thymidine (1 μCi/well; ICN, Costa Mesa, CA) for 8 h and then harvested and evaluated for [$^3$H]thymidine incorporation. Peptide-specific proliferation was determined as a difference between [$^3$H]thymidine incorporation in experimental wells and the highest negative control (medium or bovine albumin). We defined a SI as a ratio of maximal [$^3$H]thymidine incorporation in response to $Tet_{A8,30}$ during immunization to the preimmunization value for the response.

**Cytokine Release Assay.** PBL samples collected after three or six immunizations were depleted of CD8$^+$ cells using MACS MS (Miltenyi Biotec, Auburn, CA) or StemCell (Vancouver, British Columbia, Canada) separation columns. CD8-depleted cells (CD4-enriched) were cultured at $2 \times 10^5$/well in the complete medium with 10% human AB serum: (*a*) without any additional antigen; (*b*) in the presence of bovine albumin (0.5 μg/ml); (*c*) with tetanus toxoid (1:2000 dilution of vaccine prep; Wyeth-Ayerst Laboratories); or (*d*) with tetanus helper peptide $Tet_{A8,30}$ (10 μM). Cells were plated in duplicate per condition/time point. Supernatants were collected daily, days 1–5. Concentrations of IFN-γ, IL-4, and IL-10 in the supernatants were determined by ELISA using the matching antibody pairs from Endogen in accordance with the manufacturers' directions.

**Peptide-MHC Tetramer Staining.** Three peptide-MHC tetramer preparations (HLA-A*0201/YMDGTMSQV, HLA-A*0201/YLEPGPVTA, and HLA-A*0301/ALLAVGATK) were provided by National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility (Atlanta, GA). The specificity of tetramers was confirmed by titration using polyclonal CTL lines with known specificities (data not shown). Patient samples were evaluated after enrichment for CD8$^+$ cells using immunomagnetic labeling, followed by negative selection on separation columns (StemCell Technologies). Cells were stained with the phycoerythrin-labeled tetramers (1:100 dilution; 10 μg/ml), anti-CD8 antibody (APC, MHCD 0804, Caltag), and anti-TCR α/β chain antibody (FITC, 347773; Becton Dickinson, San Jose, CA). We used fluorescence-activated cell sorter Calibur (Becton Dickinson) with CELLQuest software to enumerate tetramer$^+$/TCR$^+$/CD8$^+$ cells. A tumor-free lymph node sample BRC2421 from a breast cancer patient was used as a negative control. The anti-TCR α/β antibody used has been shown not to block tetramer binding (22), and our initial assay optimization studies confirmed that (data not shown).

## RESULTS

**Patient Accrual.** Twenty-two patients were accrued in this trial in the six groups shown in Fig. 1. Most of the patients ($n$ = 15; 68%) had stage III disease, including 13 with nodal metastases and 2 with intransit metastases only. Among the 13 patients with node-positive disease, only 5 had disease limited to sentinel nodes. The remainder presented with gross palpable nodal disease, with 1–8 nodes positive (mean, 3.6; median, 2; 8, 7, 6, 2, 2, 2, 1, 1). Three patients (14%) had stage IIB disease, with primary melanomas >4 mm thick and negative nodes (2 with negative sentinel node biopsy and 1 with negative clinical exam only). Four patients (18%) had stage IV disease, including 1 with a resected lung metastasis and 3 with resected distant skin metastases, 2 from unknown primary lesions. One patient with stage III disease was entered into the protocol with small volume disease consisting of satellite metastases, but the remaining 21 patients were clinically free of disease at the time of study entry (Table 1).

*Table 1* Patient characteristics

| | *n* | % |
|---|---|---|
| Stage | | |
| IIB | 3 | 14 |
| III | 15 | 68 |
| IV | 4 | 18 |
| Age (yr) | | |
| ≤50 | 11 | 50 |
| >50 | 11 | 50 |
| Gender | | |
| Male | 12 | 54 |
| Female | 10 | 46 |
| Sites of metastasis | | |
| None (sentinel node negative, 2; sentinel node not done, 1) | 3 | 14 |
| Nodes | 13 | 59 |
| Nonpalpable lymph node metastases (N1a, 4; N2a, 1) | 5 | (23) |
| Palpable lymph node metastases | 8 | (36) |
| N2b | 2[a] | |
| N3 | 6[b] | |
| Intransit skin | 2 | 9 |
| Distant | 4 | 18 |
| Lung | 1 | |
| Distant skin (2 with unknown primaries) | 3 | |
| Prior IFN-α therapy | | |
| No | 14 | 77 |
| Failed on IFN-α | 3 | 14 |
| Did not tolerate IFN-α | 1 | 5 |
| Took 1 yr of IFN-α without failure | 1 | 5 |
| No. of patients accrued in groups | | |
| Group 1 | 4 | 18 |
| Group 2 | 5 | 23 |
| Group 3 | 4 | 18 |
| Group 4 | 2 | 9 |
| Group 5 | 4 | 18 |
| Group 6 | 3 | 14 |
| Adjuvant | | |
| QS-21 (groups 1, 3, and 5) | 12 | 54 |
| Montanide ISA-51 (groups 2, 4, and 6) | 10 | 45 |
| Peptide | | |
| $gp100_{2(M)}$ | 9 | 41 |
| $gp100_{2(M)}$ + tetP | 6 | 27 |
| Chimeric molecule | 7 | 32 |

[a] Includes 1 patient with recurrent nodal disease.
[b] Includes one patient with clinically evident disease at the time of protocol entry, in the form of multiple satellite metastases after resection of a palpable node.

Eighteen patients (82%) were entered on the protocol within 4 months of their last surgical procedure, 16 within 3 months. The others started 5, 9, 10, and 25 months after definitive surgery because of an interval trial of IFN-α (patients 1103 and 1108) or because of patient delay to consider options (patients 1117 and 1119). Five of the patients had had prior high dose IFN-α, three of whom progressed on or soon after IFN-α therapy (patients 1102, 1111, and 1118), and two who entered this trial after completing <1 month of high-dose IFN-α (1103) or a full-year of IFN-α at 3 million units/day off protocol (patient 1108). Otherwise, patients had not been treated with other therapy. Twelve patients completed the protocol's seven injections. Three patients completed six of the seven injections:

*Table 2* Patients enrolled in protocol, with clinical outcome

| Patient ID | Gender | Group | Stage | No. of vaccines | Reason off protocol | Time to progression (yr) | Site of progression | Follow-up[a] (yr) | At last follow-up | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Alive? | NED? |
| 1101 | M | 1 | III | 4 | Progression | 0.5 | Multidistant | 0.6 | N | N |
| 1102 | M | 1 | III | 7 | | | | 4.6 | Y | Y |
| 1103 | F | 2 | IIB | 7 | | | | 4.5 | Y | Y |
| 1104 | F | 2 | IV | 7 | | 1.7 | Skin | 4.6 | Y | Y |
| 1105 | F | 1 | IIB | 7 | | 2.5 | Nodes | 4.4 | Y | Y |
| 1106 | F | 2 | III | 7 | | | | 4.1 | Y | Y |
| 1107 | M | 1 | III | 2 | Progression | 0.2 | Intransit | 1.6 | N | N |
| 1108 | M | 2 | III | 7 | | | | 3.6 | Y | Y |
| 1109 | M | 2 | III[b] | 2 | Progression | 0.2 | Lung | 0.4 | N | N |
| 1110 | M | 3 | III | 7 | | 1.8 | Nodes | 3.0 | Y | Y |
| 1111 | F | 3 | III | 6 | Other cancer | | | 0.9 | Y | Y |
| 1112 | M | 6 | III | 7 | | | | 3.1 | Y | Y |
| 1113 | M | 5 | III | 6 | Noncompliance | | | 2.6 | Y | Y |
| 1114 | M | 4 | IV | 4 | Progression | 0.4 | Nodes | 1.2 | N | N |
| 1115 | M | 6 | III | 4 | Progr or MI[c] | 0.5 | Not evaluable | 0.5 | N | N |
| 1116 | F | 6 | III | 3 | Progression | 0.3 | Intransit | 0.9 | Y | N |
| 1117 | M | 3 | III | 7 | | | | 2.4 | Y | Y |
| 1118 | M | 5 | IV | 7 | | | | 2.1 | Y | Y |
| 1119 | F | 5 | IV | 7 | | 1.5 | Liver | 1.9 | Y | Y |
| 1120 | F | 4 | IIB | 7 | | | | 1.6 | Y | Y |
| 1121 | F | 5 | III | 4 | Progression | 0.5 | Intransit | 1.4 | Y | N |
| 1122 | F | 3 | III | 6 | Adverse event | | | 1.5 | Y | Y |

[a] Mean follow-up, 2.3 years; median follow-up, 2.0 years; median follow-up of living patients, 2.6 years. NED, no evidence of disease.
[b] This patient had minimal measurable disease at protocol entry.
[c] Progr or MI, progression or myocardial infarction.

1 was removed for toxicity after injection 6 (patient 1122); 1 patient failed to report for his last injection (patient 1113); and 1 because of progression of an unrelated cancer (breast cancer) requiring cytotoxic chemotherapy (patient 1111). Patients 1113 and 1122 have returned for follow-up since that time, without evidence of recurrence or of toxic sequelae. Seven patients were taken off the protocol because of tumor progression after two to four injections. All patients were followed for clinical outcome, and all patients were assessed for immunological responses except for 1, who was taken off the protocol for tumor progression after two injections. Additional clinical details are provided in Table 2.

**Toxicity and Adverse Experiences.** Most patients experienced mild and transient pain at the vaccine injection sites, but inflammation at those sites was mild enough that by the time of each subsequent vaccine, induration was usually absent. Patients often experienced very mild flu-like symptoms for ~1 day after each vaccine, but that usually occurred several days after the vaccine was administered. Individual toxicities are reported, based on NIH Common Toxicity Criteria, in Table 3. Mild toxicity was reported in 9 of 13 patients (69%) vaccinated with the gp100 peptides plus the tetanus helper peptide (groups 3–6), compared with 7 of 9 patients (77%) vaccinated with the gp100 peptide alone ($P$ = not significant; data not shown). These results are summarized in Table 4.

Both adjuvants were very well tolerated with minimal toxicity, but there were more toxicities reported with QS-21 than with Montanide ISA-51 (Tables 3 and 4). Local toxicities were observed in 9 of 12 patients vaccinated with QS-21 (75%) and in 3 of 10 patients with Montanide ISA-51 (30%). The local toxicities observed with QS-21 vaccines were generally limited to local pain at the time of vaccine injection and were of very short duration. By $\chi^2$ analysis, with correction for degree of freedom = 1, the difference in local toxicities between adjuvants does not reach significance ($\chi^2 = 2.81$; $P \sim 0.09$); however, this small sample size is not adequate to rule out differences between these two groups. Overall toxicities were higher with QS-21 as well, with one or more toxicities reported in 12 of 12 patients (100%), compared with 4 of 10 patients (40%) with Montanide ISA-51. By $\chi^2$ analysis, with correction for degree of freedom = 1, this is significant ($\chi^2 = 7.09$; $P < 0.01$). Despite these differences, the vast majority of toxicities were limited to grade 1 and 2 toxicities, and all were transient.

One patient (no. 1122) had a transient grade 3 adverse experience after vaccination. This 23-year-old woman had received five vaccines without any adverse reaction. However, within 20 min after her sixth vaccine, she complained of pain in her right arm at the injection site (grade 2), numbness (grade 2), dizziness (grade 1), faintness, nausea (grade 2), stomach cramping (grade 2), and substernal tightness consistent with esophageal spasm (grade 3). She was assessed by the principal investigator (C. S.) and a research nurse practitioner (P. N.). She was hemodynamically stable, and oxygen saturation was 99% on room air. Physical exam was unremarkable. Her reaction was considered most consistent with an allergy, with a component of esophageal spasm. Thus, she was administered an oral antihistamine (Claritin). Her symptoms resolved within 90 min. Because this reaction raised a question of allergy to the vaccine, she did not receive a seventh and final vaccine. This was the last patient accrued into the study and was the only patient to experience a grade 3 toxicity. She was in group 3 (gp100 + QS-21 + tetanus peptide). A total of 8 patients received the

*Table 3* Toxicities observed with each adjuvant, by grade

| | QS-21 | | | | Montanide ISA-51 | | | | Total | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | None | 1 | 2 | 3 | None | 1 | 2 | 3 | None | 1 | 2 | 3 | % |
| Local skin, especially pain | 3 | 7 | 2 | | 7 | 3 | | | 10 | 10 | 2 | | 55 |
| Skin, other | 8 | 4 | | | 10 | | | | 18 | 4 | | | 18 |
| Flu-like symptoms | 10 | 2 | | | 7 | 2 | 1 | | 17 | 4 | 1 | | 22 |
| Headache | 9 | 3 | | | 9 | 1 | | | 18 | 4 | | | 18 |
| Other pain | 10 | 2 | | | 10 | | | | 20 | 2 | | | 9 |
| Stomatitis | 12 | | | | 9 | 1 | | | 21 | 1 | | | 5 |
| Esophageal spasm | 11 | | | 1 | 10 | | | | 21 | | | 1 | 5 |
| Other gastrointestinal | 10 | 1 | 1 | | 9 | 1 | | | 19 | 2 | 1 | | 14 |
| Hematological | 11 | 1 | | | 10 | | | | 21 | 1 | | | 5 |
| Syncope | 11 | 1 | | | 10 | | | | 21 | 1 | | | 5 |
| Other neurological | 11 | | 1 | | 9 | 1 | | | 20 | 1 | 1 | | 9 |
| Dyspnea | 12 | | | | 9 | 1 | | | 21 | 1 | | | 5 |
| Endocrine | 12 | | | | 9 | | 1 | | 21 | | 1 | | 5 |
| Urinary | 12 | | | | 9 | 1 | | | 21 | 1 | | | 5 |
| Any | 0 | 9 | 2 | 1 | 6 | 3 | 1 | | 6 | 12 | 3 | 1 | 73 |

*Table 4* Summary table of maximum grade toxicity per patient, by vaccine group and by adjuvant

| | Maximum grade toxicity | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| Groups 1 and 2 | 2 | 6 | 1 | 0 | 0 | 0 |
| Groups 3–6 | 4 | 6 | 2 | 1 | 0 | 0 |
| QS-21 | 0 | 9 | 2 | 1 | 0 | 0 |
| Montanide ISA-51 | 6 | 3 | 1 | 0 | 0 | 0 |
| Total | 6 | 12 | 3 | 1 | 0 | 0 |

gp100 peptide with QS-21 (group 1+3), and 6 patients received the mixture of gp100 and tetanus peptide (group 3+4), with only this one transient grade 3 toxicity. Thus, the maximal tolerated dose was not reached for any of the components with the regimens used.

Four patients died with melanoma progression after completing this protocol or after being taken off the protocol for disease progression (Table 2). One patient developed tumor progression and neurological symptoms and lethargy associated with tumor progression (patient 1114), and 1 developed progression of a different cancer (breast cancer, patient 1111). These are not included in the toxicity listings above. In addition, one patient died while on this protocol. He developed symptoms suspicious for tumor progression, with marked lactate dehydrogenase elevation (~1500), and was scheduled for restaging studies and, because of known cardiac disease, for a cardiology evaluation within 3 days, but he died before those studies could be performed, probably because of either a myocardial infarction or tumor progression. This death was reported to the FDA because he died while enrolled in the protocol; however, his death occurred ~3 months after his fourth injection; therefore, it was not related temporally to any of his vaccines and is considered treatment unrelated and is not included in the toxicity assessment above. His disease was unevaluable but is considered a failure in terms of disease progression and mortality.

One additional patient, 1105, tolerated the vaccine protocol well but developed cutaneous lupus erythematosis almost 2 years after completing her vaccines. This was diagnosed by a skin biopsy and positive antinuclear antigen titer, and this was temporally associated with nodal recurrence of her melanoma close to the site of her skin changes. The lupus has not progressed and has required no therapy. No other autoimmune disease been observed in follow-up of these patients, except that 2 patients developed mild depigmentary skin changes during the protocol.

Because retinal pigment epithelium contains gp100, we evaluated specifically whether there was any ocular toxicity, by measuring visual acuity and performing opthalmoscopic exams at each visit, and by performing complete fundoscopic evaluation by an ophthalmologist including a fluorescein retinal angiogram. There was no evidence of visual defect or of loss of pigmented retinal epithelium on any of these patients.

**CTL Responses to Vaccination with $gp100_{280}$ Peptide.** Blood was collected at study entry and prior to each vaccine. Thus, blood was available 1 month after each of the first three injections and 3 months after each of subsequent injections. In multiple ELISPOT assays, we found no detectable CTL responses to the vaccinating peptide when the PBLs were assayed directly without *ex vivo* culture. We increased sensitivity of the assays by sensitizing the PBLs with $gp100_{280}$ peptide *in vitro*. In early studies, we sensitized with peptide once on day 0 and then evaluated T-cell response after 14 days in culture but found that more reliable results could be obtained with an ELISPOT for IFN-γ-secreting $CD8^+$ cells, performed on cryopreserved lymphocytes sensitized twice with peptide $gp100_{280}$ and then assayed on day 14. Patients were considered to have evidence of a T-cell response if reactivity to the immunizing peptide was greater than the response to both of two negative controls and was at least 4-fold greater than the prevaccine reactivity. Responses to the $gp100_{280}$ peptide were observed in 3 of 21 evaluated patients (14%), one each from group 1 (patient 1105), group 2 (patient 1108), and group 4 (patient 1120). Examples of data for two patients are shown in Fig. 2, *A* and *B*. For these 2 patients, responses were also evident after a single *in vitro* sensitization (data not shown). Assays for IFN-γ secretion corroborate ELISPOT findings as shown in Fig. 2, *C* and *D*. These

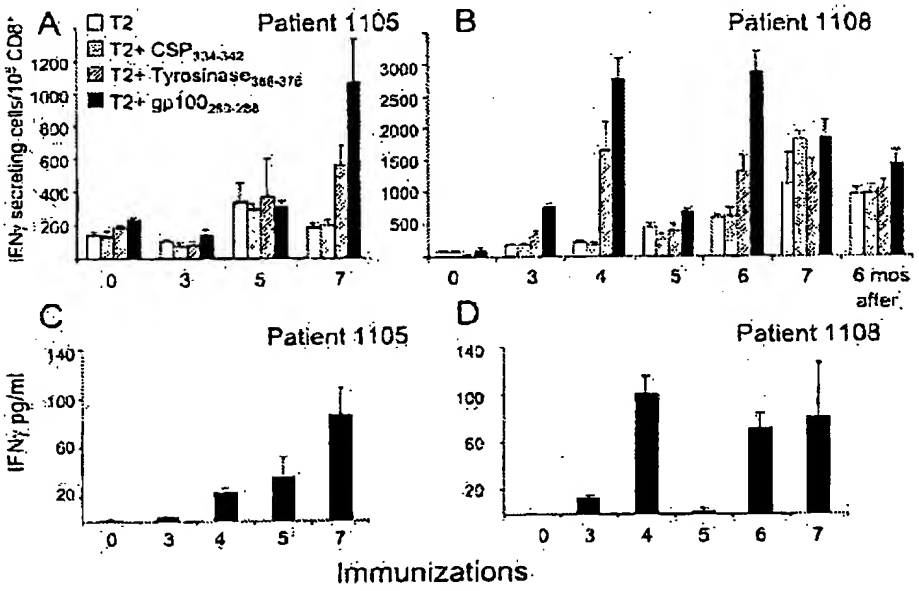


*Fig. 2* Detection of CTL responses to vaccination. PBLs were collected prior to vaccination and 1 or 3 months after each vaccination and were cryopreserved. Subsequently, PBLs from each of several representative dates were cultured in parallel, with weekly sensitizations with the vaccinating peptide, $gp100_{280}$ (YLEPGPVTA) × 2. The first stimulation used peptide alone; the second stimulation used peptide presented on autologous PBLs. After 14 days from the initiation of culture, $CD8^+$ cells represented about 20–25% of the cells. $CD8^+$ cells were then purified by negative selection on StemCell columns and were evaluated in ELISPOT assays for reactivity to the gp100 peptide pulsed on T2 cells. Results from patient 1105 are shown in *A* and from patient 1108 in *B*. aliquots were also evaluated for secretion of IFN-γ, measured by ELISA assay in 24-h supernatants. IFN-γ secretion by PBLs in response to T2 + $gp100_{280}$ is shown for patients 1105 (*C*) and 1108 (*D*), with background reactivity to T2 alone subtracted. Background reactivity to T2 ranged from 7.7 to 53.7 pg/ml (mean, 21.6) for patient 1105 and from 2 to 24 pg/ml (mean, 3.9) for patient 1108. *Bars*, SD.

data support the conclusion that the peptide YLEPGPVTA is immunogenic *in vivo* in humans, but that the immune response detected in the peripheral blood is not striking either in its magnitude or its frequency.

**CTL Recognizing $gp100_{280}$ Accumulated in Lymph Nodes 2 Years after Vaccination.** One of these patients with a T-cell response evident in the PBLs (patient 1105) developed tumor progression in a regional inguinal node 1.5 years after completing this vaccine protocol. She underwent surgery (by C. S.) to remove the two tumor-involved nodes and the remainder of nodes in the inguinal nodal basin. She has since done well for ~2 additional years after surgery and remains clinically free of disease. Thus, this was an isolated nodal recurrence after vaccination.

The $CD8^+$ T cells infiltrating this tumor deposit were evaluated directly *ex vivo* (without culture or antigen exposure) using MHC-tetramers for HLA-A2/YLEPGPVTA. CTL recognizing YLEPGPVTA were identified as 0.24% (1 of 417) of the $CD8^+$ cells in that node (Fig. 3*A*). CTLs reactive to another A2-restricted melanoma peptide (YMDGTMSQV from tyrosinase), with which this patient was not vaccinated, were not observed. CTL recognizing the gp100 peptide were evident also in a tumor-free node from the same nodal basin but at lower frequency (Fig. 3*A*).

**CTLs Recognizing $gp100_{280}$ in Tumor Metastasis Fail to Secrete IFN-γ in Response to Peptide *ex Vivo*.** Lymphocytes from the metastatic tumor-involved node were evaluated by ELISPOT for their ability to secrete IFN-γ in response to the peptide $gp100_{280}$. No IFN-γ-secreting cells were identified above background (Fig. 4*B*). However, there was high spontaneous release of IL-10 among lymphocytes from peripheral blood (~1:500) at the time of the patient's lupus diagnosis and tumor recurrence, and there was also spontaneous nonspecific release of IL-10 by cells in her tumor-involved node (Fig. 4*A*).

To determine whether the lymphocytes in the tumor-involved node could be activated *in vitro* by the autologous metastatic melanoma cells, lymphocytes and tumor cells from the tumor-involved node were cocultured in IL-2 (20 units/ml)-containing medium for 14 days and then evaluated by ELISPOT for reactivity to the gp100 peptide. This approach has been effective in a prior study for detection of CTL responses to an HLA-A3-restricted gp100 peptide (23). However, in the present analysis of this patient's tumor, no YLEPGPVTA-specific cells were found, suggesting either a failure of the metastatic tumor to present the gp100 peptide or a tolerizing effect of the tumor on YLEPGPVTA tetramer-positive T cells (data not shown). The original primary tumor was diffusely positive for gp100 expression and tyrosinase expression by immunohistochemistry, but the metastatic tumor cells in the inguinal node were positive for

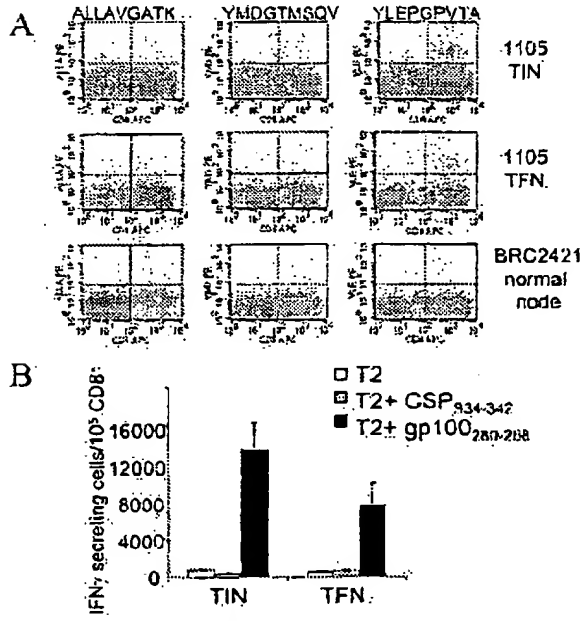


*Fig. 3* Peptide-reactive CTLs identified in tumor deposit 1.5 years after vaccination. *A*, T cells infiltrating tumor of patient 1105 and T cells from a negative node were evaluated directly *ex vivo*, after enrichment for $CD8^+$ cells by negative selection on a StemCell column, and were tested with MHC tetramers HLA-A2/YMDGTMSQV, HLA-A2/YLEPGPVTA, and HLA-A3/ALLAVGATK. A normal node from a patient with breast cancer was used as a negative control. HLA-A2/YLEPGPVTA$^+$ cells are 0.24% of $CD8^+$ cells in the tumor-involved node (*TIN*) and 0.08% in the tumor-free node (*TFN*), with all negative controls being 0.02–0.04%. *B*, lymphocytes infiltrating a tumor-involved node from patient 1105, 1.5 years after completing vaccination, were stimulated with the immunizing peptide (gp100) weekly × 2. $CD8^+$ cells were selected and then evaluated by ELISPOT at 14 days. *Bars*, SD.

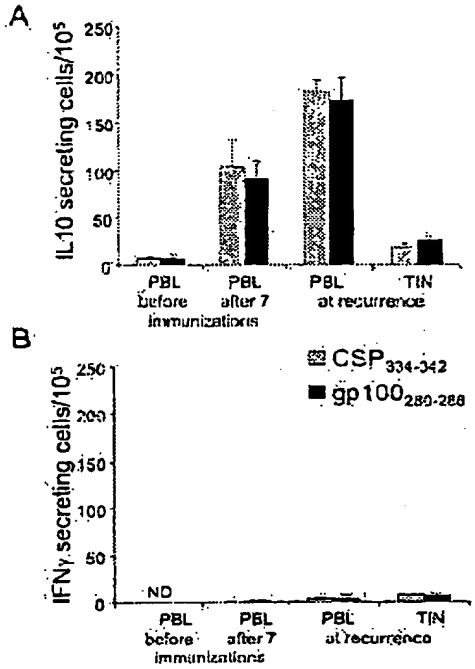


*Fig. 4* *Ex vivo* analysis of patient 1105's tumor-infiltrating lymphocytes and PBLs for secretion of IFN-γ or IL-10. A cryopreserved specimen from the tumor-involved node and PBLs of patient 1105 were thawed and then evaluated in two parallel ELISPOT assays, one with a readout of IFN-γ and another for enumeration of IL-10-secreting cells. Quantitation was performed as described. We tested the prevaccination PBL sample, PBLs collected 6 months after the last vaccine, and the PBL sample collected 1 year later, at 1 week after the patient presented with a rash and enlarged inguinal node. *TIN* data represent tumor-involved node from a surgical resection of inguinal nodes. *Bars*, 1 SD of triplicate wells. *ND*, not done because of a limited sample.

gp100 expression in only 50% of cells, and the expression was weak, whereas expression of tyrosinase continued to be evident in virtually all cells, many at moderate to strong intensity (not shown).

However, $CD8^+$ T cells producing IFN-γ in response to the $gp100_{280}$ peptide were detected in this tumor-involved node after two *in vitro* sensitizations with peptide (Fig. 3*B*). In addition, gp100-reactive T cells were similarly evident in a lymph node in the same inguinal basin, which was not involved with tumor. The magnitude of the response is more than 10 times higher than that observed in the postvaccination blood sample after a comparable *in vitro* sensitization. Over 13% of $CD8^+$ cells recognized this gp100 peptide after two *in vitro* sensitizations, representing an ~60-fold increase from the proportion of tetramer$^+$ cells in the *ex vivo* sample.

**Th1 Responses Induced by Modified Tetanus Helper Peptide.** We included a helper peptide from tetanus in this vaccine, which we modified to improve its stability. An $NH_2$-terminal alanine residue was added to prevent cyclization of the $NH_2$-terminal glutamine residue in $Tet_{830\ 844}$. The resulting 16-mer peptide AQYIKANSKFIGITEL ($Tet_{A830}$) has been incorporated in this Phase I trial, either as a mixture with the gp100 peptide or as part of a fusion peptide incorporating both the gp100 and tetanus peptide as a single molecule. PBLs from patients on this trial were collected before immunization, after three and after six immunizations. Samples from 20 patients were evaluated for their ability to proliferate upon the addition of the tetanus peptide directly to the PBLs (without any additional *ex vivo* sensitization). Peptide-specific proliferation was determined as a difference between [$^3$H]thymidine incorporation in experimental wells and the highest negative control (nothing or bovine albumin). We defined a SI as a ratio of maximal [$^3$H]thymidine incorporation in response to $Tet_{A830}$ to the preimmunization value. Examples are shown in Fig. 5. A SI of 4 or greater is considered positive. We found that 3 of 5 patients (60%) in the group treated with the mixture of peptides and 6 of 7 patients (86%) in the group treated with chimeric fusion peptide responded to the tetanus peptide as a result of immunization (total, 75%; Table 5). In contrast, a response to the tetanus peptide was detected in only 1 of 8 patients vaccinated with $gp100_{280}$ peptide alone. These data, summarized in

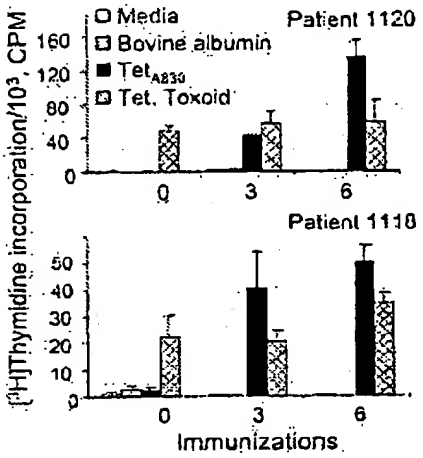


*Fig. 5* Proliferative responses to tetanus helper peptide. PBLs of patients on the Mel16 trial were incubated with $Tet_{AK30}$ peptide for 5 days and then assayed for proliferation as measured by uptake of tritiated thymidine. PBLs were evaluated from prior to study entry (0 immunizations), 1 month after three vaccines (3 immunizations), and 3 months after 6 vaccines (6 immunizations). Representative data are shown for patient 1120 (*top*), who was vaccinated with the gp100 peptide and the tetanus helper peptide in Montanide ISA-51 adjuvant and for patient 1118 (*bottom*), who was vaccinated with the chimeric peptide gp100/tetanus, in QS-21 adjuvant. Responses are shown to medium alone, to bovine albumin as a negative control, to $Tet_{AK30}$ peptide, and to tetanus toxoid as a positive control. *Bars*, SD.

*Table 5* Proliferative response of CD4$^+$ PBLs to $Tet_{AK30}$ peptide

SI values for all patients evaluated are shown in the table. Positive responses are those with a SI ≥4. The mean increases in reactivity were 3-, 108-, and 21-fold for the three groups. The results from patients with CTL responses to the gp100 peptide are shown in bold and were: patient 1120, who had a strong helper response; patient 1108, who had no helper response but was not vaccinated with the helper peptide; and patient 1105, who also was not vaccinated with the helper peptide.

| Vaccinating peptide and adjuvant | SI | | % of positive response to $Tet_{AK30}$ |
|---|---|---|---|
| | Positive | Negative | |
| $gp100_{280-288}$ | | | |
| Montanide ISA-51 | | 3.6; 3; 2; 1; 1 | 13 |
| QS-21 | 14 | 1; 0 | |
| Mixture of $gp100_{280}$ and $Tet_{AK30}$ | | | |
| Montanide ISA-51 | 403; 118 | | 60 |
| QS-21 | 16 | 1; 0 | |
| Chimera $gp100_{280}$–$Tet_{AK30}$ | | | |
| Montanide ISA-51 | 48; 25 | 1 | 86 |
| QS-21 | 31; 26; 10; 8 | | |

Table 5, demonstrate that the modified tetanus helper peptide AQYIKANSKFIGITEL is immunogenic in humans when administered with melanoma peptides.

To assess the phenotype of the T-helper responses induced to the tetanus peptide, we evaluated cytokines produced by postvaccination PBLs from 5 patients with proliferative responses to $Tet_{AK30}$. In each case, IFN-γ was secreted in response to $Tet_{AK30}$, but neither IL-4 nor IL-10 was detected. Two examples are shown (Fig. 6). Thus, vaccination with $Tet_{AK30}$ helper peptide appears to induce Th1-type helper T-cell responses. The impact of these helper T-cell responses on the generation of CTLs cannot be assessed in a meaningful way in this small study.

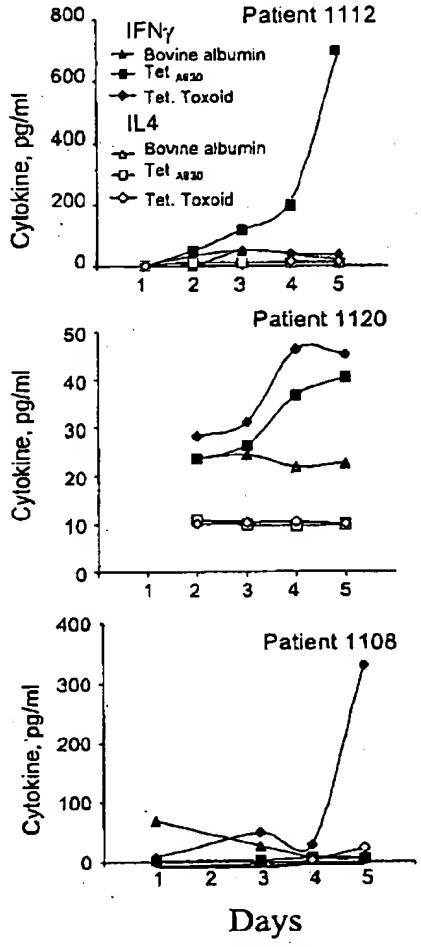


*Fig. 6* Th1 cytokine secretion in CD4$^+$ T cells responding to tetanus peptide. PBLs, enriched for CD4$^+$ cells by CD8 depletion, were plated in the presence of bovine albumin (negative control), $Tet_{AK30}$, or tetanus toxoid. Supernatants were collected daily for 5 days and were evaluated for IFN-γ, IL-4, and IL-10. There was no detectable IL-10; therefore, these data are not shown. Data for IFN-γ and IL-4 secretion are plotted for PBL samples from 2 patients (1112 and 1120) who had proliferative responses to $Tet_{AK30}$ and for PBLs from 1 patient (1108) who was not vaccinated with $Tet_{AK30}$, as a negative control. Positive controls were assessed for all three cytokines, using phorbol 12-myristate 13-acetate and ionomycin (not shown).

**DTH Responses and Skin Test Recall Responses.** All patients were tested for reactivity to standard skin test recall antigens, and reactivity was evaluated at 24 and 48 h. Any tests with induration of at least 10 mm at 24 or 48 h are considered positive. At study entry, reactivity was observed to at least one

of these recall antigens in 18 of 22 patients (82%), including trichophyton (36%), tetanus toxoid (73%), Candida (41%), and PPD (9%). After 6–12 months, 16 patients were retested with positive reactions observed in a similar percentage of patients (50, 69, 38, and 13%, respectively). There was significant inconsistency in reactivity before and after study entry. Considering all four antigens together in the 16 patients ($n = 64$) evaluated both prior to vaccination and after study entry, reactivity was observed only prevaccine in 11 cases, only postvaccine in 12 cases, both prevaccine and after study entry in 15 cases, and absent at both time points in 26. Among the 3 patients who developed CTL reactivity to the gp100 peptide, two had reactivity to one or more recall antigens, whereas one did not.

DTH responses to the $gp100_{280}$ peptide were observed in 4 patients but did not correlate with CTL reactivity measured *in vitro* in the same patients. Two patients without *in vitro* reactivity to the peptide developed DTH responses after vaccination (patients 1104 and 1111). One patient had reactivity prevaccination and then had none after vaccination, despite evidence of CTL reactivity *in vitro* (patient 1105), and 1 patient had reactivity prevaccine but was not evaluated subsequently because she was taken off protocol for disease progression (patient 1116).

**Evaluation of Adjuvants QS-21 and Montanide ISA-51.** Responses to the helper peptide were observed in four of five patients (80%) who were vaccinated with that peptide in Montanide adjuvant and in five of seven patients (71%) who were vaccinated with that peptide in QS-21 adjuvant (Table 5). The mean SI was 119 for patients vaccinated with Montanide ISA-51 adjuvant and 13 for the patients vaccinated with QS-21 adjuvant. The SI is numerically higher for the Montanide patients, but in this small sample size, the difference is not statistically significant by a two-tailed *t* test ($P > 0.18$).

Among the 3 patients with CTL responses, 2 were vaccinated with the Montanide ISA-51 adjuvant (patient 1108, group 2; and patient 1120, group 4), and 1 was vaccinated with the QS-21 adjuvant (patient 1105, group 1). Thus, there is no meaningful difference, and each adjuvant was associated with at least one CTL response to the gp100 peptide.

**Clinical Outcome.** The Kaplan-Meier estimates of survival and disease-free survival for patients in this trial, from the date of protocol entry, is 86% at 1 year (95% CI, 72–100%), and 75% beyond 1.6 years (95% CI, 56.6–94.4%), with continued follow-up to 4.7 years, without additional mortality (data not shown). Disease-free survival estimates at 1 and 2 years are 68% (95% CI, 48.7–87.6%) and 57% (95% CI, 34.8–78.5%), respectively. The estimate at 2.5 years and beyond is 49% (95% CI, 24.8–72.3%), with continued follow-up to 4.7 years, without additional progression. The survival and disease-free survival estimates for patients in this trial compare favorably with outcomes reported for patients who received high-dose IFN-α (HDI) on Eastern Cooperative Oncology Group trials E1684 and E1690, in which 4-year survival rates were approximately 49 and 55%, respectively (24, 25). Patients who remain alive without disease include 3 followed ≥4 years, 2 followed ≥3 years, and 3 followed >2 years. For the 15 patients who completed the 12-month vaccine protocol without clinical evidence of disease, 100% remain alive, and 73% have been free of recurrence. All four patients who had recurrences had isolated solitary metastases [cutaneous, lymph node ($n = 2$), and liver] that were resectable, and all remain clinically free of disease (at 5+, 14+, 23+, and 35+ months).

## DISCUSSION

In this Phase I trial of peptide vaccination with the $gp100_{280-288}$ peptide and a modified tetanus helper peptide, we evaluated toxicity and cellular immune responses. The findings are that the toxicity was limited to mild (grade I/II) local toxicity and mild (grade I/II) systemic toxicity in the vast majority of patients. All toxicities were transient. The toxicities were more prevalent for the QS-21 adjuvant than for the Montanide ISA-51 adjuvant but were not increased by the addition of the tetanus helper peptide. The toxicities were acceptable for both adjuvants and for all three peptides evaluated for the patients enrolled in this study.

The study was designed to test also for any evidence of autoimmune reactivity against normal melanocytes or normal retinal pigment epithelium. Vitiligo was not a convincing part of the patient outcome, but there was some very mild depigmentation in 2 patients that appeared to increase mildly during vaccination. There was no evidence of injury to retinal pigment epithelium either as manifested in visual acuity changes or as detected by detailed fundoscopic examination. Thus, vaccination with the gp100 peptide with or without a tetanus helper peptide, in Montanide ISA-51 or QS-21, is well tolerated with minimal negative impact on quality of life. This type of vaccine approach is markedly less toxic than what has been reported with high-dose IFN-α (24, 26–28).

The biological intent of vaccination with the $gp100_{280}$ peptide was to induce gp100-reactive CTLs in these patients. We detected CTL responses in PBLs of 3 of 21 evaluable patients (14%). These responses were not detectable in *ex vivo* samples but required sensitization *in vitro* with peptide before the responses were evident. Regardless, they were absent prior to vaccination and were increased after several vaccinations. Furthermore, the ELISPOT data were corroborated by measures of IFN-γ released directly into the medium. Thus, this is convincing evidence that vaccination with the $gp100_{280}$ peptide in adjuvant does induce T-cell responses that are detectable in the peripheral blood of some patients.

The low frequency and magnitude of CTL responses to $gp100_{280}$ peptide may be attributable to any of several factors, including the level of immunogenicity of this gp100 peptide, peptide stability, the total adjuvant effect, preexisting tolerance in patients with melanoma, or low sensitivity for detection of responses. The naturally processed $gp100_{280}$ peptide induces CTL responses naturally in some patients with melanoma, and our experience is that CTLs reactive to this peptide can be induced from most HLA-$A2^+$ melanoma patients by repeated *in vitro* stimulation of lymphocytes with HLA-$A2^+$ $gp100^+$ melanoma cells (2). Thus, there is ample evidence that immune responses to this peptide can occur when the antigen is presented effectively. When vaccinating with peptide, the intent is for the peptide to bind directly to the relevant class I MHC molecule on antigen-presenting cells in the skin, where the most relevant cells are epidermal and dermal dendritic cells (Langerhans cells). In this trial, we vaccinated into the s.c. tissue. Short

peptides, including $gp100_{280}$, have a very short half-life in fresh human plasma, suggesting that naturally occurring peptidases in the skin and plasma of humans will degrade them very rapidly (29). Migration of dendritic cells to sites of inflammation in response to the adjuvant may take several hours, and the half-life of this peptide *in vivo* may be shorter than that required for dendritic cells accumulation. Vaccination with peptide in adjuvant may be more effective when administered intradermally.

Another possible explanation for the low rate and magnitude of CTL responses to the $gp100_{280}$ peptide may be related to the approaches used for immune monitoring. In animal models of CTL responses to viral vaccines or viral infection, the peak time for CTL reactivity in the spleen is near the end of the first week after vaccination (30). This peak reactivity falls rapidly over the following weeks, leaving a small population of memory T-cells, which will proliferate promptly upon restimulation. In the present study, we drew blood for CTL assays just prior to each vaccine, which was 1–3 months after the prior vaccine. Thus, we suspect that the acute CTL response to vaccination likely peaked at 1 week and may have fallen to a much lower level by the time we had drawn blood in many of the patients. There were two patients with reactivity to the gp100 peptide by DTH, and these patients did not have evidence of CTL reactivity *in vitro*. Comprehensive assessment of CTL responses to vaccination may require more intensive evaluations of multiple lymphoid compartments, rather than evaluation of the blood lymphocytes alone.

Other studies have also detected low responses to vaccinating peptides (31, 32). Thus, the finding in the present study of CTL reactivity to the $gp100_{280}$ peptide in 14% of patients likely underestimates both the frequency and magnitude of responses. For future studies, we believe it will be more accurate to measure CTL responses in PBLs at 1-week intervals and to measure CTL responses also in a lymph node draining a vaccine site and in tumor deposits when available.

In one patient, T cells recognizing the $gp100_{280}$ peptide were detectable in a tumor-involved node and a tumor-free node, directly *ex vivo* by use of MHC-peptide tetramers. Thus, vaccination with that peptide may have induced a CTL response that persisted long after completion of the vaccine protocol (Fig. 3). T cells recognizing another HLA-A2-restricted epitope (tyrosinase$_{368\ 376D}$) peptide were not found. Despite the presence of T-cells recognizing melanoma antigens, melanoma recurred in lymph node tissue, and we are interested in determining the mechanisms that permitted immune escape by these tumor cells.

Immunohistochemical evaluation of the lymph node metastasis suggests that the tumor cells may have partially down-regulated gp100 expression, compared with the primary melanoma. This is unlikely to explain immune escape completely. It is illustrative that stimulation of the lymphocytes with tumor *in vitro*, in IL-2, does not lead to generation of $gp100_{280}$-reactive CTLs, suggesting that the tumor cells in this metastasis may not present that peptide, despite expression of gp100. A possible explanation would be a defect in antigen processing or MHC class I molecule down-regulation.

**This patient also developed cutaneous lupus at the same** time that she developed this tumor recurrence, presenting with a rash in the same body region. Lupus is associated with spontaneous secretion of IL-10 by circulating lymphocytes and with cellular immune dysfunction (33, 34). Furthermore, IL-10 blockade can restore cellular immune function of lupus patients (34, 35). The peripheral blood and node of this patient contained significant numbers of IL-10-secreting cells, which may well have an impact on cellular immune function as well as on the activation state of $gp100_{280}$-specific CTLs, as manifested by their inability to produce IFN-γ.

The fact that this was an isolated recurrence and that the patient has remained disease free since that time may represent some immunological control of tumor progression. It is interesting that all of the recurrences observed in patients who completed the protocol (at least six vaccines) have been isolated recurrences that were amenable to surgical resection, whereas most patients with melanoma progress in multiple sites, as did the patients who progressed early on this protocol.

The tetanus helper peptide AQYIKANSKFIGITEL has been modified to stabilize the glutamine residue at the $NH_2$ terminus by addition of the new $NH_2$-terminal alanine (A) residue. To our knowledge, this modified form of the peptide has not been used in other trials. The trial was designed such that patients were randomized to receive the tetanus peptide or not to receive it. As shown in Table 5, proliferative responses to the tetanus peptide were obtained in 86% of patients who received the chimeric fusion peptide $gp100_{280}$-$Tet_{A830}$ and in 60% of those who received the gp100 and tetanus peptides as separate species. IFN-γ was produced by PBLs in response to the tetanus peptide, but IL-4 and IL-10 were not detectable. Thus, this modified tetanus peptide appears to induce Th1-type helper T-cell responses reliably in the majority of patients.

The patients on this trial were skin-tested with standard recall antigens, including tetanus toxoid, but this did not result in increases in T-helper responses to tetanus toxoid after vaccination. Instead, the responses to the tetanus toxoid protein remained fairly constant during the vaccine regimen (Fig. 5). Interestingly the response to the tetanus peptide $Tet_{A830}$ does not correlate with the response to tetanus protein, suggesting that this peptide may not represent the dominant epitope from tetanus toxoid or may not be processed and presented in all HLA-DR settings. Regardless, helper T-cell responses to this peptide cannot be explained simply by changes in reactivity to tetanus toxoid but appear to be induced by vaccination with the peptide.

In summary, vaccination with an HLA-A2-restricted YLEPGPVTA peptide ($gp100_{280}$) induced CTL responses to that peptide that were detectable in 14% of patients. We believe that evaluation of PBLs only and collection of PBLs 1 month or more after each vaccine diminished the sensitivity of this assessment. We conclude that this peptide can induce CTL responses *in vivo*, but that neither the route of administration nor the approach to immune monitoring has been optimized. Tumor-infiltrating lymphocytes reactive to $gp100_{280}$ were identified in a metastatic tumor deposit that arose after vaccination, suggesting that CTLs responding to $gp100_{280}$ were induced by vaccination and are capable of migrating to tumor deposits and may persist long-term. However, in this patient, the CTLs recognizing that peptide were dysfunctional *in vivo*. Other studies have identified CTL in patients that specifically recognize a defined tumor antigen but that fail to respond appropriately to the antigen, suggesting tolerance induction or anergy (36, 37). Ad-

ditional work is needed to understand the range of etiological factors for these immune escape mechanisms.

Some patients treated on this protocol have had recurrences, and some of those patients have died with progressive metastatic melanoma, but the overall survival of patients on this protocol is excellent and compares favorably with the outcome of similar-stage patients treated with high-dose IFN-α, based on two recent Eastern Cooperative Oncology Group studies. This peptide should be evaluated further in more aggressive vaccination regimens and with more comprehensive monitoring approaches.

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# Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma

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**The cloning of the genes encoding cancer antigens has opened new possibilities for the treatment of patients with cancer. In this study, immunodominant peptides from the gp100 melanoma-associated antigen were identified, and a synthetic peptide, designed to increase binding to HLA-A2 molecules, was used as a cancer vaccine to treat patients with metastatic melanoma. On the basis of immunologic assays, 91% of patients could be successfully immunized with this synthetic peptide, and 13 of 31 patients (42%) receiving the peptide vaccine plus IL-2 had objective cancer responses, and four additional patients had mixed or minor responses. Synthetic peptide vaccines based on the genes encoding cancer antigens hold promise for the development of novel cancer immunotherapies.**

The identification of the genes encoding melanoma-associated antigens has opened new possibilities for the immunotherapy of patients with cancer[1,2]. To identify melanoma antigens involved in tumor rejection in humans, we utilized tumor-infiltrating lymphocytes (TILs) grown from metastatic melanoma nodules and adoptively transferred to the autologous melanoma patient[3,4]. Those TILs associated with *in vivo* tumor regression were used to screen tumor-derived cDNA libraries to identify the antigens they recognized. Two antigens, MART-1 and gp100, whose recognition was restricted by HLA-A2*0201 (HLA-A2) were initially identified, and both were shown to be nonmutated differentiation antigens expressed by cells of melanocytic lineage including melanomas, normal melanocytes and pigmented retinal cells, but not in other normal tissues or nonmelanoma tumors[5,6]. By screening large numbers of peptides from these two molecules conforming to known HLA-A2 binding motifs, a single, immunodominant, nine amino acid peptide was identified in the MART-1 molecule, and five different epitopes recognized by TILs were identified in the gp100 molecule[7,8].

The MART-1 epitope, m27–35, and two gp100 epitopes, g209–217 and g280–288, bound to the HLA-A2 molecule with intermediate affinity and did not have optimal amino acids at one of the known MHC-binding anchor residues. We thus studied a large number of synthetic peptides in which single- and double-amino acid substitutions were introduced at HLA-A2 binding positions[9]. A modified g209–217 peptide (referred to as g209-2M), in which a methionine replaced the natural threonine at position 2, bound to the HLA-A2 molecule with greater affinity than the unmodified peptide and was shown to have an increased ability to generate melanoma-reactive cytotoxic T lymphocytes (CTLs) *in vitro* when used for sensitization of peripheral blood mononuclear cells (PBMCs) from patients by using techniques previously described[10–14]. Because these *in vitro* studies suggested a relation between immunogenicity and the MHC binding affinity of the peptide from this nonmutated self protein, we have now conducted a clinical study in which HLA-A2$^+$ patients with metastatic melanoma were immunized with the synthetic peptide, g209-2M. A variety of immunization strategies using peptides have been evaluated in animal models[15–24]. For the present clinical trial, we selected a method of immunization involving emulsification of peptide in incomplete Freund's adjuvant (IFA) injected every 3 weeks, because of the success of this schedule in the induction of cell-mediated immune responses in mice[16]. In our previous trials using unmodified peptides, we saw no evidence of a dose response between 0.1 mg and 10 mg, and we therefore selected the intermediate dose of 1 mg for the current trial[13,14].

Few previous studies of the immunization of melanoma patients with melanoma peptides have been performed. Hu *et al.* treated patients with a MAGE-1 peptide pulsed onto antigen-presenting cells and presented evidence of immunization in lymphocytes obtained from tumor nodules or at the vaccination site, although it required three *in vitro* restimulations to detect this response[25]. Jaeger *et al.* injected nine melanoma patients with a combination of peptides derived from the MART-1, tyrosinase, and gp100 proteins injected intradermally weekly for 4 weeks[26,27]. Following vaccination, *in vitro* evidence of immunization against peptides could be detected in the PBMCs of three of six patients against the MART-1 peptide, two of six patients against the tyrosinase peptide and in none of six patients receiving the gp100 peptide. An initial *in vitro* stimulation and one restimulation with peptide were required for detecting these activities. Marchand *et al.* injected a MAGE-3 peptide in 14 patients with melanoma, and although two showed a partial tumor regression, lymphocytes reactive with the peptide could not be detected in these patients after immunization[28]. We previously immunized 23 patients with an im-

munodominant peptide from the MART-1 antigen and could detect an increase in antipeptide precursors in PBMCs, but three repetitive *in vitro* stimulations were required for detecting this reactivity[13].

As shown in the present clinical study of patients immunized with the modified g209-2M peptide, PBMCs obtained from these patients after, but not before, immunization exhibited a high degree of reactivity against the native g209–217 peptide, as well as against HLA-A2⁺ melanoma cells. The administration of the g209-2M peptide, along with adjuvant IL-2, mediated tumor regression in 42% of patients with metastatic melanoma.

Table 1 Specificity of reactivity against g209–217 peptide and HLA-A2⁺ melanomas

| No. of immunizations | *In vitro* sensitization with peptide* | Stimulator (pg IFN-γ/ml) T2 | T2(280) | T2(flu) | T2(209) | 501 mel (A2⁺) | SK23 mel (A2⁺) | 624.28 mel (A2⁻) | 888 mel (A2⁻) |
|---|---|---|---|---|---|---|---|---|---|
| None | 209-2M | 135 | 102 | 230 | 146 | 195 | 180 | 89 | 115 |
| | 209 | 118 | 90 | 238 | 84 | 148 | 231 | 162 | 261 |
| | flu | 178 | 123 | 35,570† | 124 | 290 | 284 | 172 | 282 |
| 2 | 209-2M | 86 | 74 | 61 | 24,150 | 72,780 | 43,250 | 124 | 33 |
| | 209 | 86 | 62 | 165 | 9,890 | 25,710 | 19,480 | 104 | 98 |
| | flu | 121 | 118 | 36,460 | 132 | 313 | 448 | 85 | 362 |

*Day 11 after culture with 1 μg/ml peptide; PBMCs were tested for IFN-γ release after culture with tumor or T2 cells pulsed with peptide.
†In this and subsequent tables values greater than 100 pg IFN-γ/ml and at least twice that of all controls are underlined.

### Clinical characteristics of the patients

Nine patients received the g209–218 peptide in IFA, 11 received the g209-2M peptide in IFA, and 31 received the g209-2M peptide in IFA plus systemic IL-2. Eighty-three percent of patients were between the ages of 31 and 60, and all had good performance status (ECOG 0 or 1). All patients had extensive metastatic disease, and many were heavily pretreated. All had undergone prior surgery, and many had received prior chemotherapy, radiotherapy or immunotherapy sometimes including IL-2; 82% of patients had received two or more different treatments.

### Specificity of the immunologic assay

The assessment of immune reactivity of PBMCs was based on the ability to generate specific antipeptide and antitumor reactivity following *in vitro* exposure of PBMCs to the immunizing peptide (see Methods). The *in vitro* sensitization assay was highly specific. As seen in a characteristic experiment shown in Table 1, PBMCs before immunization exhibited no *in vitro* sensitization against peptide after 11 days of incubation with the g209–217 or the g209-2M peptide, but developed specific anti-influenza reactivity after incubation with the control flu peptide. However, post-immunization PBMCs obtained from the patient following two injections of the g209-2M peptide in IFA could be specifically sensitized *in vitro* to the native g209–217 peptide as well as to the flu peptide. Reactivity of these *in vitro* sensitized cultures was also seen against two HLA-A2⁺ melanomas (501-mel and SK23-mel), but not against two HLA-A2⁻ melanomas (624.28-mel and 888-mel).

### Immunization with the g209–217 peptide in IFA

The reactivity of PBMCs from eight of the nine patients immunized with the native g209–217 peptide in IFA before and after two immunizations is shown in Table 2 (sufficient lymphocytes were not obtained from patient 5). Only two of eight patients showed reproducible evidence of immunization to the native g209–217 peptide (patients 4 and 6), and one of these patients (patient 6) exhibited a low level of immune reactivity against the g209–217 peptide before immunization.

### Immunization with the modified 209-2M peptide in IFA

Peripheral blood mononuclear cells from all 11 patients before immunization with the modified 209-2M peptide in IFA failed to show reactivity to peptide-pulsed T2 cells (Table 3). However, following immunization with the modified 209-2M peptide in IFA, 10 of 11 patients showed a consistently high level of immunization against the native g209–217 peptide, but not against the control g280–288 peptide. All patients were tested after two immunizations except patient 1 who was tested after one immunization. Only patient 3 consistently failed to show any evidence of reactivity following immunization. Multiple independent experiments were performed on separate aliquots of PBMCs, and the results of replicate consecutive experiments on these 11 patients are shown in Table 3.

To test the reactivity of PBMCs against tumor cells, PBMCs obtained from patients before and

Table 2 Reactivity of PBMCs from patients immunized with g209–217 peptide in IFA

| Patient | Expt.† | Before immunization Assay stimulator* (pg IFN-γ/ml) T2 | T2(280) | T2(209) | T2-(209-2M) | After immunization Assay stimulator* (pg IFN-γ/ml) T2 | T2(280) | T2(209) | T2(209-2M) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 85 | 94 | 77 | 82 | 61 | 68 | 69 | 89 |
| | 2 | 36 | 30 | 47 | 43 | 22 | 36 | 152 | 72 |
| | 3 | 128 | 142 | 88 | ND | 52 | 45 | 72 | ND |
| 2 | 1 | 264 | 234 | 221 | 276 | 514 | 362 | 341 | 381 |
| | 2 | 246 | 167 | 179 | ND | 113 | 83 | 61 | ND |
| 3 | 1 | 291 | 253 | 273 | 362 | 107 | 113 | 97 | 128 |
| | 2 | 49 | 33 | 41 | ND | 51 | 35 | 138 | ND |
| 4 | 1 | 60 | 52 | 74 | 46 | 114 | 90 | 283 | 414 |
| | 2 | 74 | 74 | 56 | ND | 76 | 143 | 814 | ND |
| 6 | 1 | 69 | 45 | 392 | 516 | 152 | 150 | 4355 | 5282 |
| | 2 | 15 | 19 | 931 | ND | 26 | 21 | 4379 | ND |
| 7 | 1 | 15 | 13 | 11 | ND | 91 | 56 | 43 | ND |
| 8 | 1 | 85 | 69 | 78 | ND | 84 | 97 | 91 | ND |
| 9 | 1 | 126 | 122 | 134 | 151 | 105 | 103 | 142 | 176 |
| | 2 | 67 | 85 | 104 | ND | 46 | 68 | 38 | ND |

*PBMCs incubated with g209-2M peptide for 11 to 13 days before assay against T2 cells alone or pulsed with 1 μg/ml peptide.
†All patients tested after two immunizations. Patient 5 not tested because insufficient PBMCs were available.

after immunization with g209-2M in IFA were sensitized *in vitro* against the g209-2M peptide and tested against T2 cells pulsed with the native peptides, as well as against HLA-A2⁺ and HLA-A2⁻ melanoma cells (Table 4). In this representative experiment, all five patients showed significant post immunization reactivity against 1 μM of the native g209–217 peptide pulsed onto T2 cells. Post-immunization PBMCs from four of the five patients exhibited reactivity against T2 cells pulsed with $10^{-2}$ μM native peptide, and one patient recognized T2 cells pulsed with $10^{-1}$ μM peptide. Post-immunization PBMCs from four of the patients recognized both HLA-A2⁺, but not the two HLA-A2⁻ melanomas. The recognition of peptide-pulsed T2 cells as determined by the level of specific interferon-γ (IFN-γ) secretion correlated directly with the recognition of melanoma cells in 41 independent cultures from multiple patients ($P < 0.001$). Thus, cultures reactive with the native peptide also were capable of reacting with HLA-A2⁺ melanoma lines, although reactivity against the melanomas was often less than that against the T2 cells pulsed with peptide. Current studies of *in vivo* and *in vitro* immunization are aimed at generating T-cell receptors with the high affinity required to react strongly with the low concentrations of peptide present on melanomas.

Table 3 Reactivity of PBMCs from patients immunized with 209-2M peptide in IFA

| | | Before immunization | | | After immunization* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Assay stimulator[1] (pg IFN-γ/ml) | | | | | | | |
| Patient | Expt. | T2 | T2(280) | T2(209) | T2-(209-2M) | T2 | T2(280) | T2(209) | T2(209-2M) |
| 1 | 1 | 21 | 22 | 12 | 20 | 42 | 37 | 6,897 | 57,060 |
| 2 | 1 | 58 | 56 | 66 | 50 | 54 | 48 | 1,851 | 4,012 |
| | 2 | 1 | 4 | 2 | ND[2] | 30 | 22 | >1,000 | ND |
| 3 | 1 | 33 | 26 | 33 | 35 | 46 | 53 | 56 | 54 |
| | 2 | 145 | 127 | 124 | ND | 49 | 36 | 46 | ND |
| 4 | 1 | 133 | 123 | 184 | 213 | 40 | 35 | 2,631 | 6,086 |
| | 2 | 50 | 36 | 41 | 43 | 45 | 52 | 1,618 | 3,751 |
| | 3 | 351 | 299 | 501 | 470 | 86 | 825 | 944 | 1,057 |
| 5 | 1 | 28 | 30 | 21 | 24 | 41 | 35 | 4,366 | 6,402 |
| | 2 | 154 | 156 | 152 | 142 | 128 | 129 | 295 | 323 |
| | 3 | 29 | 18 | 37 | 32 | 27 | 22 | 856 | 1,126 |
| 6 | 1 | 38 | 28 | 24 | 31 | 44 | 47 | 152 | 671 |
| | 2 | 154 | 166 | 210 | 153 | 128 | 72 | 662 | 887 |
| | 3 | 96 | 61 | 117 | 127 | 22 | 14 | 2,374 | 5,407 |
| 7 | 1 | 44 | 66 | 72 | 82 | 104 | 81 | 4,424 | 5,411 |
| | 2 | 10 | 8 | 13 | 16 | 197 | 224 | 1,293 | 1,583 |
| | 3 | 127 | 105 | ND | 120 | 61 | 67 | 1,244 | ND |
| 8 | 1 | ND | ND | ND | ND | 17 | 25 | 845 | ND |
| | 2 | 1553 | 562 | 719 | ND | 79 | 78 | 2,326 | ND |
| | 3 | ND | ND | ND | ND | 43 | ND | 1,768 | ND |
| 9 | 1 | 345 | 337 | 355 | ND | 209 | 183 | 2,253 | ND |
| | 2 | 13 | 13 | 10 | ND | 229 | 262 | 1,550 | ND |
| | 3 | 1434 | 816 | 513 | ND | 495 | 517 | 2,408 | ND |
| 10 | 1 | 247 | 283 | 413 | ND | 29 | 39 | 1,271 | ND |
| | 2 | 135 | 102 | 146 | ND | 86 | 74 | 24,150 | ND |
| | 3 | 117 | 147 | 150 | ND | 6 | 9 | 39,690 | ND |
| 11 | 1 | 53 | 53 | 56 | ND | 65 | 71 | 154 | ND |
| | 2 | 46 | 50 | 47 | ND | 29 | 39 | 63 | ND |
| | 3 | ND | ND | ND | ND | 87 | 83 | 205 | ND |

*Patient 1 after one immunization; all other patients after two immunizations.
[1]Day 11 to 13 after culture with 209-2M peptide, PBMCs were tested for IFN-γ release following 24-h incubation with peptide-pulsed T2 cells.
[2]Not done.

Although we had previously attempted to measure precursor frequencies against the immunodominant peptides using PBMCs obtained before immunization, we were never able to do so successfully because of the very low frequency of reactive cells. Limiting dilution assays were thus performed on pre- and post-immunization PBMCs from patients 2, 5 and 7, who received two, two and four immunizations with the g209-2M peptide, respectively. Preimmunization precursor frequencies against the native g209–217 peptide were less than 1/30,000, whereas, after immunization with g209-2M peptide, the precursor frequency against the native peptide was 1/5,900, 1/2,800 and 1/3,300, respectively. These experiments thus demonstrated that patients immunized with the g209-2M synthetic peptide in IFA consistently developed high levels of circulating immune precursors reactive with the native g209–217 peptide and with tumor.

**Immunization with the 209-2M peptide in IFA plus interleukin-2**

Nineteen patients with metastatic melanoma received the g209-2M peptide in IFA followed either the next day (14 patients) or beginning 5 days later (5 patients) with a single cycle of 720,000 IU/kg IL-2 intravenously every 8 hours to tolerance. After two peptide immunizations plus IL-2, only 3 of these 19 patients (16%) developed immune reactivity in circulating PBMCs against the g209–217 peptide, as measured in the cytokine-release assay compared with 10 of 11 (91%) patients who received 209-2M peptide in IFA without IL-2 ($P < 0.0001$).

Twelve additional patients received g209-2M peptide in IFA alone [or as part of other studies with granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-12] for at least two cycles before moving to therapy with the g209-2M peptide in IFA plus IL-2. Precursors in these patients that developed as a result of their initial immunization with peptide without IL-2 continued to be detected in PBMCs following the administration of peptide plus IL-2 (data not shown).

**Clinical response in patients receiving the peptide vaccines**

One of nine patients who received the g209–217 peptide in IFA experienced an objective cancer regression that lasted 4 months. None of the 11 patients who received the g209-2M peptide in IFA experienced an objective cancer regression, although 3 patients exhibited mixed responses with complete or partial regression of some lesions.

Of the 19 patients that received the g209-2M peptide in IFA concomitantly with IL-2, 8 patients (42%) exhibited an objective cancer regression. Three additional patients experienced mixed responses,

and three others experienced stable disease, two of whom subsequently underwent complete resection of their metastatic sites. Of the 12 additional patients who received at least two cycles of g209-2M peptide in IFA without IL-2 followed subsequently by g209-2M peptide in IFA plus IL-2, we saw that 5 (42%) experienced objective cancer regression and one additional patient had a mixed response. Objective regression was seen of metastases in the brain, lung, liver, lymph nodes, muscle, skin and subcutaneous tissues. Details of the patients who experienced clinical responses are shown in Table 5 and Fig. 1.

Except for mild, transient erythema and induration at the injection site seen in some patients, there were no side effects associated with peptide injection in IFA, and all were treated as outpatients. One of the 31 patients receiving peptide plus IL-2 died of aspiration pneumonia in the midst of repeat treatment after attaining an ongoing partial response. Other patients treated with IL-2 experienced the usual side effects associated with its administration and returned to baseline within days after stopping IL-2 administration. Despite the generation of immune responses to the nonmutated "self" sequences, no ocular or aural autoimmune manifestations were seen in any patient.

Thus, in both groups of patients receiving the g209-2M peptide plus IL-2, 42% of patients experienced an objective cancer regression, often of bulky metastatic disease (Table 5, Fig. 1). In our prior experience with the use of this regimen of high-dose bolus IL-2 alone in 134 consecutive patients with melanoma, a response rate of 17% was seen[29]. In 62 melanoma patients receiving this regimen of high-dose IL-2 at our institution in other studies not involving peptide immunization, but during the same time interval as the study reported here, the response rate was 15%. Although the dangers of comparison with retrospective or simultaneous nonrandomized patient groups are well appreciated, the 42% objective response rate we have seen in patients receiving the g209-2M peptide plus IL-2 is substantially higher than what we or others have seen using IL-2 alone.

Table 4 Reactivity against tumor cells of PBMCs from patients before and after immunization with 209-2M peptide in IFA

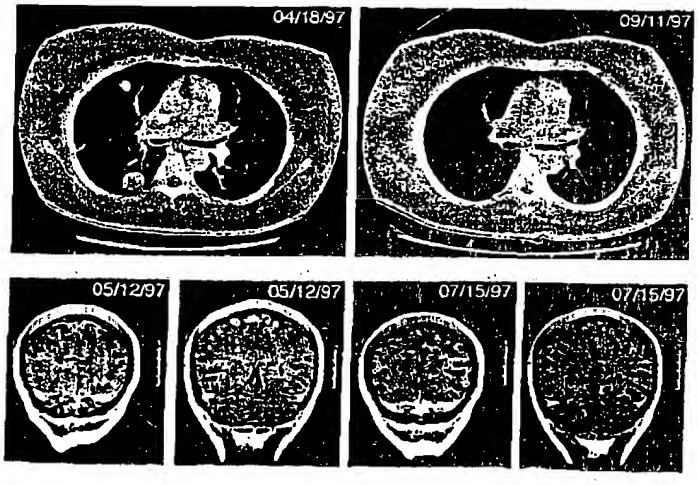
| Patient | No. of Immunizations | Stimulator* (pg IFN-γ/ml) T2 (280) | T2 (209) | T2 (A2⁺) | SO1-mel (A2⁻) | SK23-mel (A2⁻) | 888-mel (A2⁻) | 624.28-mel |
|---|---|---|---|---|---|---|---|---|
| 7 | 0 | 169 | 175 | 220 | 28 | 72 | 84 | 51 |
| | 2 | 209 | 243 | 2,555 | 1,211 | 2,037 | 98 | 60 |
| 8 | 0 | 528 | 691 | 729 | 70 | 640 | 933 | 806 |
| | 4 | 202 | 284 | 13,600 | 11,580 | 14,720 | 408 | 489 |
| 9 | 0 | 13 | 13 | 10 | ND | ND | ND | ND |
| | 4 | 229 | 590 | 3,987 | 676 | 889 | 291 | 235 |
| 10 | 0 | 117 | 147 | 150 | 19 | 90 | 39 | 42 |
| | 4 | 15 | 18 | 24,040 | 23,860 | 21,580 | 2 | 4 |
| 11 | 0 | 46 | 50 | 47 | 11 | 39 | 14 | 17 |
| | 4 | 29 | 30 | 106 | 5 | 43 | 4 | 10 |

*PBMCs incubated with g209-2M peptide for 13 days and then tested for reactivity to tumors and to T2 cells pulsed with 1μM of the g209–217 peptide on the central g280–288 peptide.
†Not done

## Discussion

The development of effective means for immunizing patients against their growing cancer is a major goal of studies in human tumor immunology. A variety of shared tumor-associated antigens present on melanomas represent possible candidates for such immunization[1,2]. The gp100 antigen was selected for evaluation because of its recognition by TILs, whose adoptive transfer was highly associated with tumor regression in patients with melanoma[8]. In prior phase I studies, patients were immunized with escalating doses of one of the immunodominant gp100 peptides (g154–162, g209–217, g280–288). The immunologic reactivity of patients before and after immunization was evaluated by *in vitro* sensitization of PBMCs to the peptide pulsed onto antigen-presenting cells[10–14]. Patients receiving the g209–217 and g280–288 peptides, but not the g154–162 peptide, developed specific immune precursors, although multiple *in vitro* sensitizations were necessary to elicit reproducible immune responses[14]. In *in vitro* studies, a synthetic modification of the g209–217 peptide (called 209-2M), which exhibited increased binding to HLA-A2 molecules, had an increased ability to generate melanoma-reactive CTLs after multiple stimulation of the PBMCs of HLA-A2⁺ patients[9]. Because of these findings, we conducted the current study in which patients were immunized with this modified peptide.

The present study makes two major points. It represents the first time that a self-peptide (in this case a synthetic modification designed to increase MHC binding) has provided a consis-



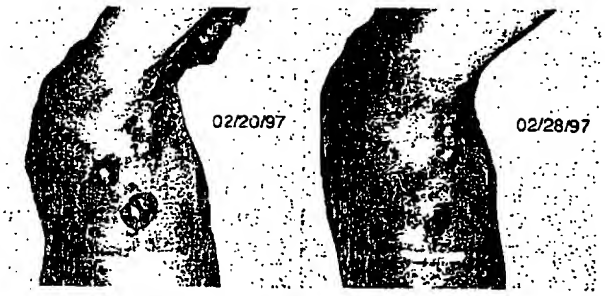




Fig. 1 Cancer regression in patients receiving immunization with 209-2M peptide in IFA plus IL-2. *top left*, Regression of multiple lung metastases in a 47-year-old female (patient I in Table 5). *bottom left*, Regression of two brain metastases in a 42-year-old female (patient k). This patient had four brain metastases all of which underwent significant regression. *above*, Regression of multiple cutaneous and subcutaneous metastases in a 44-year-old male (patient c). Significant regression was seen by 8 days after starting treatment.

tent and powerful means of immunizing patients to generate lymphocyte precursors against growing tumor. In addition, immunization with this peptide plus IL-2 appears to provide significantly higher cancer regression rates than those seen with either agent alone.

In the present study, 2 of 8 patients that received the native g209–217 peptide, compared with 10 of 11 patients immunized with the modified g209-2M peptide, developed highly reproducible reactivity against the native g209–217 peptide and against melanoma cells ($P = 0.006$). Of importance was the ability to detect this reactivity after a single exposure of PBMCs to peptide without the need for any restimulation *in vitro*. Thus, in contrast to our prior studies with unmodified peptides[18–19] and the reported studies mentioned above, the degree of immunization was substantially higher using the g209-2M peptide than had previously been seen. Significant reactivity to the native g209–217 peptide was seen within 4 days after exposure to peptide *in vitro* and continued for at least 18 days in culture (data not shown).

Further evidence for the strong immunization against the native g209–217 peptide came from studies of the precursor frequencies present in circulating PBMCs. In our prior studies and those of others, it has rarely been possible to measure precursor frequencies against melanoma antigens because of their very low frequency[30–32]. In the present series, precursor frequencies against the native peptide were less than 1 in 30,000 (the lower limit of detection in this assay) PBMCs before immunization compared with frequencies between 1 in 2800 and 1 in 5900 after immunization. In these patients, therefore, precursor frequencies were of the same magnitude often seen against viral or allogeneic antigens[33,34].

The mechanism by which immunization with the g209-2M modified peptide in IFA resulted in high levels of circulating cellular immunity against the native peptide, as well as against melanoma cells, is unclear. The emulsification of peptide in adjuvant is thought to provide prolonged exposure to antigen and to activate nonspecific inflammatory cells and possible recruitment of antigen-presenting cells to the site of immunization. Nonspecific recruitment of helper cells at the sites of vaccination or at regional draining sites may provide the necessary help to stimulate immune reactions. It is known, however, that specifically reactive $CD8^+$ cells can be generated following immunization with MHC class I-restricted peptides in the absence of $CD4^+$ cells[22,35]. The decrease in circulating precursor cells when IL-2 was administered with peptide may be due to activation of these cells and traffic to the tumor site, and attempts to reisolate these cells from the tumor are in progress.

Despite the induction of high levels of these tumor-reactive cells following immunization with the g209-2M peptide in IFA alone, none of the 11 patients in this study experienced an objective tumor response, although several patients had mixed responses with shrinkage of some lesions. Several possibilities exist to explain this paradox, although it is likely that tumor cells do not contain the appropriate costimulatory or adhesion molecules required to activate the resting precursor cells that circulate in peripheral blood as a result of immunization. Peripheral anergy may thus result from contact with antigen in the absence of costimulation. An additional source of helper function may be required. In experimental systems, provision of helper epitopes, as well as cytokines such as IL-2 or IL-12, have been shown to increase the immunizing capacity of MHC class I-reactive antigens[20,21,23,36–38].

Table 5 Characteristics of patients exhibiting an objective response

| Patient | Age (yr)/Sex | Site of tumor | Type | Response Duration (mo) |
|---|---|---|---|---|
| a | 48/M | Lung | CR | 6 |
| b | 51/M | Lung<br>Subcutaneous | PR | 6 |
| c | 44/M | Lymph node<br>Lung<br>Subcutaneous<br>Cutaneous | PR | 2+ |
| d | 45/F | Lymph node<br>Bone<br>Subcutaneous | PR | 4 |
| e | 42/F | Cutaneous<br>Subcutaneous<br>Liver | PR | 7+ |
| f | 41/F | Lung | PR | 5+ |
| g | 39/M | Lung | PR | 6 |
| h | 22/F | Lung hilum | PR | 5 |
| i | 48/M | Cutaneous | PR | 6 |
| j | 43/M | Subcutaneous | PR | 5+ |
| k | 42/F | Lung<br>Lymph node<br>Liver<br>Brain | PR | 2 |
| l | 47/F | Lung<br>Lymph node | PR | 5+ |
| m | 59/M | Subcutaneous | PR | 3+ |

Although the 42% response rate seen in the present study using the peptide vaccine plus IL-2 appears higher than the 17% response rate seen in prior studies using IL-2 alone[39], randomized trials to evaluate the efficacy of g209-2M peptide immunization plus IL-2 are required. Several modifications to attempt to improve upon these results are in progress. We have recently initiated a clinical trial in which patients with melanoma are immunized simultaneously with four separate peptides (g209-2M, g280-9V, MART-1:27–35 and tyrosinase:369–377) from three different melanoma antigens (gp100, MART-1 and tyrosinase), all recognized by TIL cells associated with tumor rejection. In another trial, PBMCs from patients after immunization with g209-2M in IFA are being activated by antigen and IL-2 *in vitro* and used for adoptive transfer to the autologous tumor-bearing patient.

This study demonstrates that it is possible, in patients with metastatic cancer, to increase significantly the number of lymphocyte precursors reactive with normal nonmutated differentiation proteins such as gp100 by immunization with synthetic high binding peptides in IFA and to mediate tumor regression. Many cancers such as those that occur in the breast, prostate and ovary express proteins unique to, or overexpressed by, the tissue of origin of the tumor, and these differentiation proteins may also be suitable targets for immunotherapy.

## Methods

**Peptides.** Each of the peptides utilized in this study was prepared under Good Manufacturing Practice (GMP) conditions by Multiple Peptide Systems (San Diego, CA): g209–217, ITQVPFSV; g209-2M, IMQVPFSV; g280–288, YLEPGPVTA. The identity of each of the peptides was confirmed by mass spectral analysis. The peptides were >98% pure as assessed by high-pressure liquid chromatography analysis and had an endotoxin level less than 0.1 endotoxin units per milliliter. Each of the peptides was supplied as a white powder soluble in water.

Cultured cell lines. The melanoma cell lines 501-mel (HLA-A2$^+$), 624-38-mel (HLA-A2$^+$), 888-mel (HLA- A2$^+$), 624-28-mel (HLA-A2$^+$) were established in the Surgery Branch, NCI. The melanoma line SK23-mel (HLA-A2$^+$) was supplied by T. Boon at the Ludwig Institute for Cancer Research, Brussels, Belgium. These tumor lines and the T2 cell line (HLA-A2$^+$, TAP-deficient T-B cell hybrid) were maintained in continuous culture in medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin.

Clinical protocol. All patients had histologically confirmed metastatic melanoma and underwent a complete clinical evaluation including measurements and X-rays of all evaluable tumor sites. All patients were confirmed to be HLA-A*0201$^+$ by using high-resolution nested sequence polymerase chain reaction subtyping, and all signed an informed consent before treatment. No patient had received any treatment in the prior month nor were they receiving any immunosuppressive drugs including steroids. Before treatment, patients underwent a leukapheresis. PBMCs were isolated by Ficoll-Hypaque separation and were cryopreserved at $10^8$ cells/vial and stored at −180 °C.

Three sequential clinical trials were performed. In the first and second trials, either the native g209–217 peptide (9 patients) or the modified g209-2M peptide (11 patients) at 1.5 mg in 1.5 ml was mixed with an equal volume of incomplete Freund's adjuvant (IFA; Montanide ISA-51, Seppic, France) and spun vigorously in a vortex mixer for 12 min to form an emulsion. Two aliquots of 1 ml each were injected into the subcutaneous tissue of the anterior thigh (total peptide injection of 1 mg). All treatments were performed in an outpatient clinic. Patients received from two to six immunizations at 3-week intervals and were leukapheresed to cryopreserve PBMCs 3 weeks after every other immunization, and clinical assessment of tumor status was performed at these times as well.

In the third trial (31 patients), IL-2 (Cetus-Oncology Division, Chiron Corp, Emeryville, CA) at a dose of 720,000 IU/kg (corresponding to 120,000 Cetus units/kg) was administered as an intravenous bolus over 15 min starting either 1 or 5 days after the peptide injection. Recombinant IL-2 was provided as a lyophilized powder and was reconstituted with 1.2 ml of sterile water per vial. Vials also contained 5% mannitol and approximately 120 µg of SDS per mg of IL-2. The IL-2 was diluted in 50 ml of normal saline containing 5% human serum albumin for infusion. Patients received IL-2 every 8 hours until grade 3 or 4 toxicity was reached that could not be easily reversed by standard supportive measures. After two cycles of peptide plus IL-2, occasional cycles involved peptide alone if additional time for recovery from IL-2 side effects were required. IL-2 was routinely administered on a general surgical ward, although some patients were transferred to an intensive care unit for monitoring or for the administration of vasopressors. All patients received concomitant medications, including acetaminophen (650 mg every 4 h), indomethacin (50 mg every 8 h), and ranitidine (150 mg every 12 h), to prevent some of the side effects associated with IL-2 administration.

Evaluation of response to treatment. After two and four peptide injections, all known sites of disease were evaluated. If patients showed evidence of stable or regressing disease, additional treatments were administered and similar criteria were used to decide on subsequent courses of treatment. If patients had progression of disease, no further therapy was administered as part of this protocol.

A response was considered complete if all measurable tumor disappeared. A partial response was defined as a 50% or greater decrease of the sum of the products of the longest perpendicular diameters of all lesions, lasting at least 1 month and without increase of any tumor or the appearance of any new tumor.

*In vitro* assessment of anti-g209–217 peptide and melanoma-specific reactivity. Cryopreserved PBMCs were thawed into complete medium (CM) consisting of Iscove's modified DMEM with 25 mM HEPES buffer, 10% heat-inactivated human AB serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Biofluids, Rockville, MD; Sigma, St. Louis, MO; Pel-Freez, Brown Deer, WI). Cells were washed once and resuspended at $1.5 \times 10^6$ cells/ml in 2 ml containing peptide. Two days later recombinant IL-2 (Chiron Corp.) was added to the cultures. On day 5, CM (1 ml) was withdrawn and replaced with fresh CM containing IL-2. CM (1 ml) was replaced whenever the medium became acidic. To determine the optimal conditions for assessing PBMC reactivity to the immunizing peptides *in vitro*, experiments were conducted using PBMCs obtained from patients following immunization. A variety of peptide concentrations ranging from 0.01 to 10 µM were evaluated, as were harvest times from 4 to 18 days and IL-2 concentrations from 30 IU to 300 IU per ml in the culture. On the basis of these optimization experiments (data not shown) *in vitro* assays were conducted using $3 \times 10^6$ PBMCs in 2 mls of medium incubated with 1 to 2 µM peptide with 300 IU/ml IL-2 added on day 2 and harvested between days 11 and 13 after initiation of the culture. The harvested cells were washed once in HBSS and $10^5$ cells were added in 0.1 ml to wells of flat-bottom 96-well plates. Stimulator cells consisting of $10^5$ T2 cells pulsed with peptide were added in 0.1 ml. Alternatively, $10^5$ melanoma cells were added as stimulators. To pulse T2 cells with peptide, either $10^{-4}$ µM, $10^{-2}$ µM or 1 µM peptide was incubated with $6 \times 10^6$ T2 cells in 3 ml for 3 h at 37 °C with intermittent mixing. The cells were then washed once with HBSS before addition to the responder cells. The cultures were incubated for 18 to 24 h at 37°C in 5% $CO_2$. IFN-γ release into the supernatant was measured using a standard ELISA assay.

To determine precursor frequencies, limiting dilution assays were performed by adding varying numbers of lymphocytes to flat-bottom 96-well plates in 24 replicate wells along with $10^5$ irradiated autologous PBMCs and 1 µM g209-2M peptide. IL-2 (300 IU/ml) was added to each well on day 2, and medium was changed on day 5 and whenever the medium became acidic. After 10 to 14 days, the cells were replica plated and stimulated with T2 cells alone or T2 cells pulsed with native g209–217 peptide. After overnight incubation, the supernatant was harvested and tested for IFN-γ release using a standard ELISA assay. The fraction of positive wells (>2 times IFN-γ release compared with wells stimulated with unpulsed T2 cells) was used to calculate precursor frequency using standard Poisson analysis.

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# TUMOR REGRESSIONS OBSERVED IN PATIENTS WITH METASTATIC MELANOMA TREATED WITH AN ANTIGENIC PEPTIDE ENCODED BY GENE *MAGE-3* AND PRESENTED BY HLA-A1

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**Thirty-nine tumor-bearing patients with metastatic melanoma were treated with 3 subcutaneous injections of the MAGE-3.A1 peptide at monthly intervals. No significant toxicity was observed. Of the 25 patients who received the complete treatment, 7 displayed significant tumor regressions. All but one of these regressions involved cutaneous metastases. Three regressions were complete and 2 of these led to a disease-free state, which persisted for more than 2 years after the beginning of treatment. No evidence for a cytolytic T lymphocyte (CTL) response was found in the blood of the 4 patients who were analyzed, including 2 who displayed complete tumor regression. Our results suggest that injection of the MAGE-3.A1 peptide induced tumor regression in a significant number of the patients, even though no massive CTL response was produced.** ***Int. J. Cancer*** **80:219–230, 1999.**
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Human melanoma cells bear antigens that can be recognized by autologous cytolytic T lymphocytes (CTL) (Boon *et al.*, 1994). Some of these antigens are encoded by genes of the *MAGE* family (van der Bruggen *et al.*, 1991). The *MAGE* genes are expressed in a significant proportion of tumors of various histological origins. They are not expressed in normal cells, except male germ line cells, which are devoid of major histocompatibility complex (MHC) molecules and therefore do not present MAGE antigens (Takahashi *et al.*, 1995; Tomita *et al.*, 1993; Uyttenhove *et al.*, 1997). The *MAGE*-encoded antigens are therefore tumor-specific shared antigens.

Gene *MAGE-3* codes for an antigenic nonapeptide that is recognized by cytolytic T cells on the human leukocyte antigen (HLA)-A1 molecule (Gaugler *et al.*, 1994). Analysis by reverse transcription-polymerase chain reaction (RT-PCR) amplification of a large number of melanoma samples indicated that *MAGE*-3 was expressed in 76% of metastatic melanomas and in 36% of primary melanomas (Brasseur *et al.*, 1995). In Europe, about 28% of the population carry the HLA-A1 allele. Accordingly, about 21% of the metastatic melanomas are expected to present the MAGE-3.A1 antigen.

We examined whether immunization against the MAGE-3.A1 antigen can be of therapeutic benefit to cancer patients. The objectives of our study were to define the safety and toxicity of the MAGE-3.A1 peptide, to observe the clinical evolution of tumor-bearing patients immunized with this antigenic peptide and to find out whether tumor regressions were linked to strong CTL responses. In 1995, we published a preliminary report describing 3 tumor regressions observed among 6 melanoma patients treated with subcutaneous (s.c.) injections of the MAGE-3.A1 peptide given 3 times at monthly intervals (Marchand *et al.*, 1995). To confirm these results, our study was extended to a larger group of patients. We report here the observations made on 39 melanoma tumor-bearing patients.

## SUBJECTS AND METHODS

### *Eligibility criteria*

From March 1994 to July 1997, HLA-A1-positive patients whose tumor expressed the *MAGE-3* gene were included in our study (LB 94-001) if they were older than 18 years of age and had a good performance status (WHO scale 0 or 1). The study was opened to melanoma patients with histologically proven tumors of stages III and IV, according to the American Joint Committee on Cancer staging system (AJCC, 1992). The patients described here were classified in one of the following subgroups according to the

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extent of their disease: regional disease (stage III) *N2a*, metastasis of more than 3 cm in greatest dimension in any regional lymph nodes; *N2b*, in-transit metastasis; *N2c*, both *N2a* and *N2b*. Distant metastasis (stage IV) *M1a*, metastasis in skin or s.c. tissue or lymph node(s) beyond the regional lymph nodes, and *M1b*, visceral metastasis. Patients with brain metastasis, as assessed by brain computed tomography (CT) scan, were excluded. HLA class I typing was performed on blood lymphocytes by classical serological methods. The expression of gene *MAGE-3* was assessed by RT-PCR on a small frozen tumor sample as described previously (Weynants *et al.*, 1994). No other anti-tumor treatment was given during the study. All participants gave written informed consent before entering the study and were treated as outpatients.

Thirty-nine tumor-bearing melanoma patients entered the study and their evolution is described in this report. In addition, 6 tumor-free melanoma patients and 4 tumor-bearing patients with tumors other than melanoma received the same treatment. No regression or significant stabilization was observed in any of the 4 non-melanoma tumor-bearing patients, whose evolution is not described in the present report.

*Vaccination*

The MAGE-3.A1 nonapeptide EVDPIGHLY (acetate) was synthesized by U.C.B-Bioproducts (Braine-l'Alleud, Belgium; UCB SF124, batch 1S1, issued on 10/03/1994). The lyophilized peptide was diluted in sterile, endotoxin-free phosphate-buffered saline (PBS), pH 7.4 (Boehringer Ingelheim Bioproducts) at a concentration of 100 or 300 μg/ml, and distributed in 1.5-ml vials for single injections. Vials were stored at −80°C and thawed just before injection.

The treatment consisted of 3 vaccinations at 1-month intervals. For each vaccination, the peptide was injected at a total dose of either 100 or 300 μg. The vaccine was divided between 2 s.c. sites and 2 intradermal (i.d.) sites distant from the tumor (⅘ of the dose was injected at each s.c. site, and ⅒ at each i.d. site). No injections were given into limbs where the draining lymph nodes had been surgically removed or irradiated. When possible, the injection sites were changed for each vaccination.

Some tumor-bearing patients who displayed tumor regression following 3 immunizations received additional vaccinations with the MAGE-3.A1 peptide. In such cases, the same dose and route of administration were used.

*Follow-up of patients*

Toxicity was evaluated according to the National Cancer Institute of Canada common toxicity criteria. Tumor staging was carried out at study entry and in the 2-week period following the third injection, by physical examination and CT scans of brain, chest and abdomen. For patients who displayed tumor stabilization or regression, additional tumor staging was performed at regular intervals.

Because our report was aimed at assessing all possible evidence for an effect of the treatment on tumor evolution, we describe here the tumor regressions in detail. In future studies, for which the aim will be the quantitative evaluation of the efficacy of the treatment, tumor responses will be reported according to the synthetic WHO criteria.

*Analysis of the CTL response*

The presence of anti-MAGE-3 CTL was evaluated as follows.

*Method A.* Autologous peripheral blood mononuclear cells (PBMC) were thawed, resuspended in serum-free X-VIVO 10 medium (Whittaker, Walkersville, MD), and incubated over 40 min at 4°C with magnetic microbeads coated with an anti-CD8 antibody (MACS; Miltenyi, Bergisch Gladbach, Germany). The $CD8^+$ cells were sorted with a magnet, washed, resuspended in stimulation medium consisting of Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Eggenstein, Germany) supplemented with 5% human serum, L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L) and 2-mercaptoethanol ($5 \times 10^{-5}$ M), and seeded at $10^5$ cells/well in 1-ml wells (Falcon 3078, Becton Dickinson, Mountain View, CA). Cells ($5 \times 10^5$ $CD8^+$) were seeded for patient 1 (LB-945) and $10^6$ $CD8^+$ cells for patient 7 (AVL-3). The $CD8^-$ cells were washed, resuspended in X-VIVO 10 medium containing MAGE-3.A1 peptide EVDPIGHLY or influenza.A1 peptide CTELKLSDY (100 μM), and incubated at room temperature for 60 min, during which time the cells were irradiated (30 Gy from a $^{137}Cs$ source), washed and resuspended in stimulation medium. These stimulator cells were added ($10^6$ cells/well) to the $CD8^+$ lymphocytes, and human rIL-2 (Proleukin, Chiron, Amsterdam, The Netherlands) was added at 30 U/ml (1 U/ml is the concentration that gives half-maximal proliferation of CTLL-2 cells). On day 7, the responder lymphocytes of each well were washed, resuspended in medium containing the same concentration of rIL-2 in 2-ml wells, restimulated with $10^6$ autologous PBMC pulsed with the relevant peptide and irradiated as on day 0. On day 14, restimulation was performed under the same conditions, except that peptide-pulsed C1R cells (Storkus *et al.*, 1987) transfected with an HLA-A1 construct were used as stimulators. On day 21, aliquots of the cultures were tested in a chromium-release assay against Epstein-Barr virus (EBV)-transformed B cells BM21, in the presence or absence of the relevant antigenic peptides, and in the presence of a 50-fold excess of unlabelled K562 cells to inhibit the activity of natural killer (NK)-like lytic effectors.

*Method B.* Another approach involving a large number of $CD8^+$ lymphocytes (more than $2 \times 10^7$) was carried out as described elsewhere (Chaux *et al.*, 1998). In brief, responder $CD8^+$ T cells of patient 7 (AVL-3) were distributed among 96 microcultures (226,000 cells/well). Autologous monocyte-derived dendritic cells were irradiated, incubated with 50 μM of the MAGE-3.A1 peptide EVDPIGHLY, and distributed in the microcultures (85,000 cells/well) to stimulate the autologous $CD8^+$ T cells. IL-6 (1,000 U/ml) and IL-12 (10 ng/ml) were added during the first week, followed by IL-2 (10 U/ml) and IL-7 (5 ng/ml) from day 7. The microcultures were restimulated each week with autologous phytohemagglutinin (PHA)-activated T cells (150,000 cells/well), incubated with the peptide and irradiated. On day 25, aliquots of the cultures were tested in a chromium-release assay against the HLA-A1 EBV-transformed B cell line BM21 in the presence or absence of the MAGE-3.A1 peptide, and in the presence of a 50-fold excess of unlabelled K562 cells to inhibit the activity of NK-like lytic effectors.

## RESULTS

We treated 39 HLA-A1 patients having a melanoma that expressed gene *MAGE-3*. All patients had detectable metastases. The treatment consisted of 3 vaccinations with the synthetic MAGE-3.A1 peptide at monthly intervals. For each vaccination, 7 patients received a total dose of 100 μg, 32 patients received 300 μg. Peptide was injected in 2 s.c. and 2 i.d. injection sites distant from the tumor.

*Toxicity*

No significant toxicity or side effects were observed in any of the patients. Six patients had a mild inflammatory reaction at the site of superficial metastases (grade 1 toxicity). No delayed-type hypersensitivity reactions or other inflammatory reactions were observed at the sites of i.d. injection.

*Tumor evolution*

Of the 39 patients, 14 were withdrawn after the first or the second injection because of rapid progression of the disease necessitating other forms of treatment. Of these 14 patients, 3 had regional disease and 11 had distant metastases at study entry (Fig. 1). Three patients died of tumor progression in the 4-week period following the last injection.

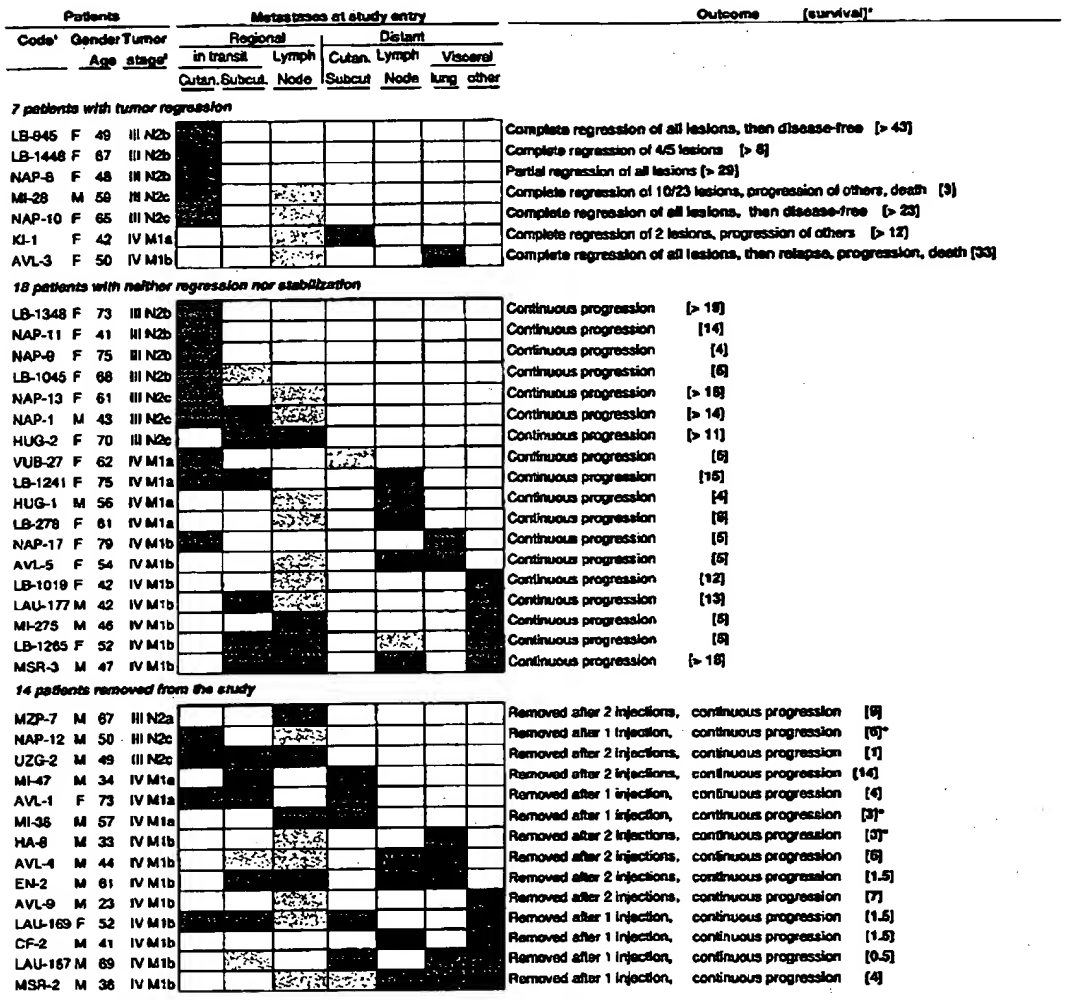



FIGURE 1 – Evolution of the melanoma tumor-bearing patients treated with peptide MAGE-3.A1.

Metastases present at study entry

Metastases removed before study entry

* Survival is shown in months after onset of treatment

* Exact date of death unknown. A minimum survival estimation is given here

1 The alphabetic part of the code indicates the recruiting center

AVL: A. Van Leeuwenhoek Huis, NCI, Amsterdam
CF: Centre Jean Perrin, Clermont-Ferrand, France.
EN: Centre hospitalier universitaire, Grenoble, France.
HA: Medizinische Hochschule Hannover
HUG: Hôpital Cantonal Universitaire de Genève
KI: Karolinska Institutet Radiumhemmet, Stockholm
LAU: LICR and CPO, Lausanne
LB: LICR and UCL -St-Luc, Brussels
MI: Istituto dei Tumori, Milano
MSR: Centro S. Raffaele, Milano
MZP: Krankenhaus Nordwest, Frankfurt
NAP: Hôtel Dieu, Nantes
UZG: Universitair Ziekenhuis, Ghent, Belgium.
VUB: AZ-VUB, Brussels

2 According to the AJCC classification (see section Subjects and Methods)

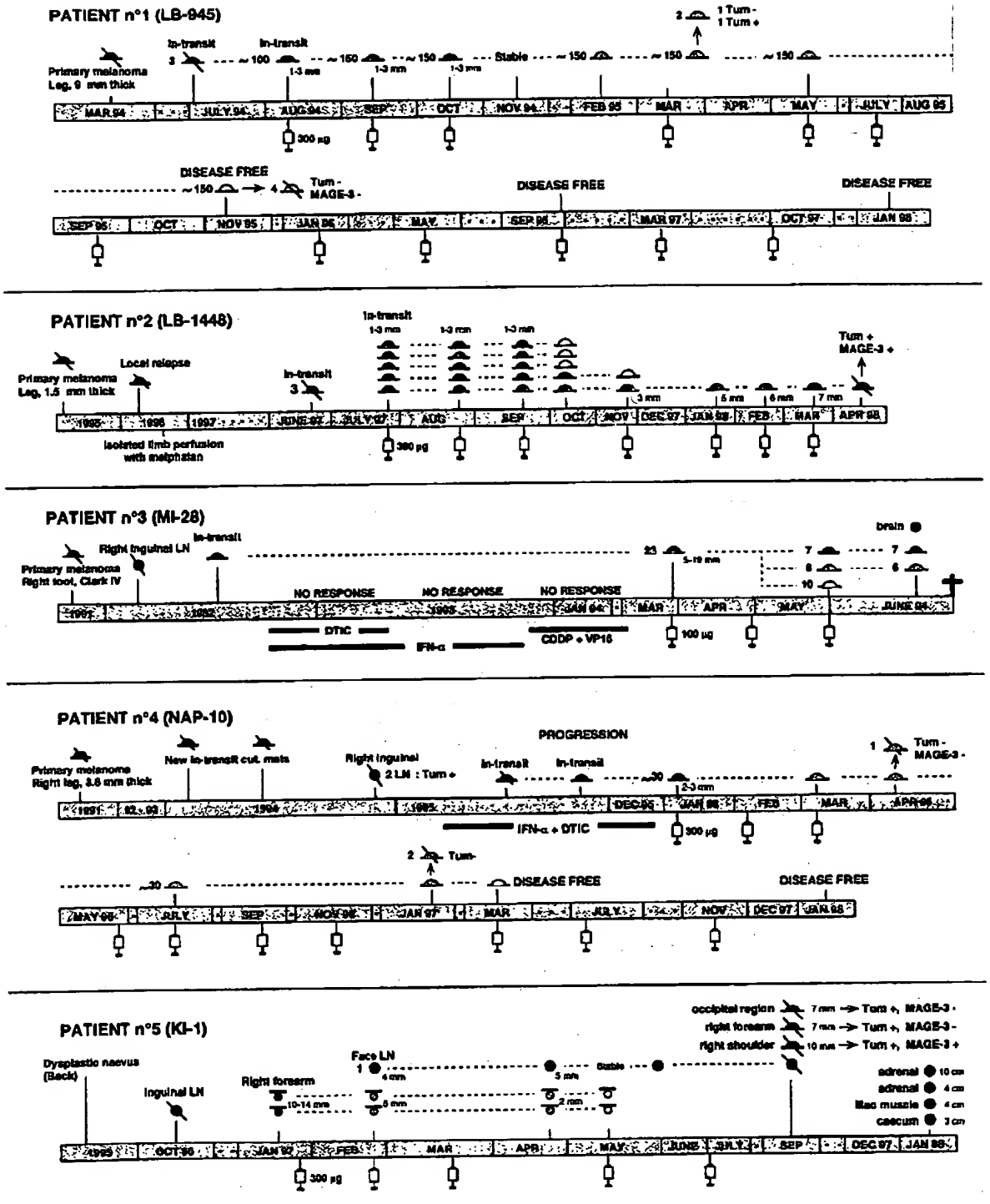

PATIENT n°1 (LB-845)
Primary melanoma
Leg, 9 mm thick
In-transit
In-transit
1 Tum -
1 Tum +
Stable
MAR 94
JULY 94
AUG 94
SEP
OCT
NOV 94
FEB 95
MAR
APR
MAY
JULY
AUG 95
300 µg
DISEASE FREE
Tum -
MAGE-3 -
DISEASE FREE
DISEASE FREE
SEP 95
OCT
NOV 95
JAN 96
MAY
SEP 96
MAR 97
OCT 97
JAN 98
PATIENT n°2 (LB-1448)
Primary melanoma
Leg, 1.5 mm thick
Local relapse
In-transit
In-transit
Tum +
MAGE-3 +
Isolated limb perfusion
with melphalan
1995
1996
1997
JUNE 97
JULY 97
AUG
SEP
OCT
NOV
DEC 97
JAN 98
FEB
MAR
APR 98
380 µg
PATIENT n°3 (MI-28)
Primary melanoma
Right foot, Clark IV
Right inguinal LN
In-transit
brain
NO RESPONSE
NO RESPONSE
NO RESPONSE
DTIC
IFN-α
CDDP + VP16
100 µg
JAN 94
MAR
APR
MAY
JUNE 94
PATIENT n°4 (NAP-10)
Primary melanoma
Right leg, 3.6 mm thick
New in-transit cut. mets
Right inguinal
2 LN : Tum +
In-transit
In-transit
PROGRESSION
1 Tum -
MAGE-3 -
IFN-α + DTIC
300 µg
DEC 95
JAN 96
FEB
MAR
APR 96
2 Tum -
DISEASE FREE
DISEASE FREE
MAY 96
JULY
SEP
NOV 96
JAN 97
MAR
JULY
NOV
DEC 97
JAN 98
PATIENT n°5 (KI-1)
occipital region
right forearm
right shoulder
7 mm → Tum +, MAGE-3 -
7 mm → Tum +, MAGE-3 -
10 mm → Tum +, MAGE-3 +
Dysplastic naevus
(Back)
Face LN
Inguinal LN
Right forearm
Stable
adrenal 10 cm
adrenal 4 cm
Iliac muscle 4 cm
caecum 3 cm
1995
OCT 96
JAN 97
FEB
MAR
APR
MAY
JUNE
JULY
SEP
DEC 97
JAN 98
300 µg

FIGURE 2

Among the 25 tumor-bearing patients who received the planned treatment of 3 vaccinations, neither regression nor stabilization was observed with 18 patients who all showed continuous progression. Seven of these patients had regional disease, whereas 11 had distant metastases (Fig. 1).

Tumor regressions were observed in 7 of the 25 patients who received the complete treatment (Fig. 1). Five of these patients had regional disease only and 2 had distant metastases. Their evolution is described below and summarized in Figures 2 and 3.

Patient 1 (LB-945, female, 49 years) had about 100 small cutaneous in-transit metastases on the left leg at study entry (Fig. 2). The patient had received no treatment other than surgery. The MAGE-3.A1 peptide (300 μg) was injected in the thighs and in the arms. By the second injection, the cutaneous nodules had become more numerous, but no further progression was observed at the time of the third injection. The stabilization was confirmed 1 month later. Four months after the third injection, all the nodules appeared flattened and dry (Fig. 4*a*). One month later, 2 nodule sites were biopsied and found to contain a large number of macrophages containing melanin. A few remaining tumor cells were observed in one site and none in the other. Because of the favorable clinical evolution, the patient received 4 additional injections of the same peptide at 2-month intervals. No other treatment was given. Fifteen months after the first injection, the patient appeared to be completely free of disease: all the skin lesions had become impalpable even though melanin tattooing remained visible at the sites of the former nodules. Biopsies of several sites revealed no tumor cells and no *MAGE-3* expression as assessed by RT-PCR. Physical examinations and CT scans showed no evidence of disease. The patient received additional injections at increasingly longer intervals. Forty-one months after the onset of treatment, she was still disease-free and the melanin tattooing had completely disappeared (Fig. 4*a*).

Patient 2 (LB-1448, female, 67 years) had undergone in 1996 surgical excision of a cutaneous local relapse on the right leg, followed by adjuvant isolated perfusion with melphalan (Fig. 2). In June 1997, 3 new in-transit cutaneous metastases appeared and were removed surgically. One nodule was tested for *MAGE-3* expression and found to be positive. One month later, the patient developed 5 new small cutaneous in-transit metastases on the right leg. Vaccination was initiated with 300 μg of MAGE-3.A1 peptide. Two weeks after the third injection, 3 of the 5 in-transit metastases had completely disappeared, the 2 remaining ones appeared to be stable, and no new metastases had appeared. Six weeks later, only 1 nodule of 3 mm diameter remained present. Two additional vaccinations were carried out at 2-month intervals. The complete regression of 4 of the 5 nodules continued 9 months after study entry, but, at that time, the 5th nodule had progressed from 3 to 7 mm diameter. It was excised and found to express gene *MAGE-3*.

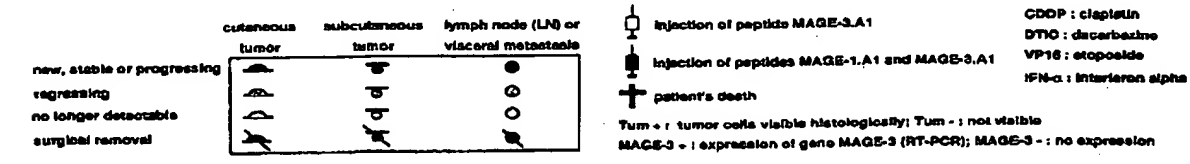
Patient 3 (MI-28, male, 59 years) had melanoma localized to the right leg with 23 cutaneous or subcutaneous in-transit metastatic nodules ranging from 5 to 19 mm diameter at study entry (Fig. 2). These nodules had not responded to several cycles of chemotherapy involving dacarbazine (DTIC), interferon-α (IFN-α) and a combination of cisplatin and etoposide. The patient was vaccinated with 100 μg of peptide, starting 4 weeks after cessation of chemotherapy. By the third injection, 10 of the 23 nodules, including the largest one, had regressed completely. Six other nodules had regressed partially. Six nodules were stable and one was progressing. Three weeks later, a brain metastasis was discovered and the patient died soon thereafter.

Patient 4 (NAP-10, female, 65 years) had a primary melanoma of the right leg removed in 1991 (Fig. 2). In 1994, in-transit metastases and 2 invaded regional lymph nodes were removed. In 1995, despite adjuvant treatment associating DTIC and IFN-α, new in-transit metastases appeared and were removed, and this treatment was interrupted by the end of the year. In January 1996, when vaccination with 300 μg of peptide was started, the patient displayed about 30 small cutaneous in-transit metastases disseminated on the right leg and thigh (Fig. 4*b*). By the third vaccination, partial flattening of all the lesions was observed. One month later, one regressing lesion was biopsied and neither *MAGE-3* expression nor tumor cells were found. By September 1996, all the lesions were almost completely flat (Fig. 4*b*). The patient received additional injections with the same amount of peptide, first at 2-month and then at 4-month intervals. Fourteen months after the onset of treatment, all the lesions were impalpable. After 24 months, the patient remained free of disease.

Patient 5 (KI-1, female, 42 years) had a suspect lesion of the back removed in 1995 (Fig. 2). In October 1996, an inguinal lymph node was removed and was found to contain melanoma cells expressing *MAGE-3*. In January 1997, 2 new s.c. metastases measuring 10 and 14 mm diameter, respectively, appeared on the right upper arm. Vaccination with 300 μg of peptide was initiated. By the second injection, the 2 s.c. metastases had reduced to 5 mm diameter, but a small metastatic maxillary lymph node had appeared. Two months after the third injection of peptide, the 2 s.c. lesions of the right arm had totally disappeared and the lymph node was stable. Two additional injections were given. Three months later, during which the lymph node had remained stable, 3 new cutaneous metastases had appeared. All the lesions were removed surgically and were found to contain tumor cells. Gene *MAGE-3* was expressed in only 1 of the 3 cutaneous lesions. Because of this tumor progression, the injections were stopped. The patient then remained disease-free for a period of 4 months, after which she relapsed with several visceral metastases.

Patient 6 (NAP-8, female, 48 years) had a primary melanoma of the left leg in 1989 (Fig. 3). Between 1990 and 1995, in-transit metastases appeared and displayed continuous slow progression, despite repeated treatments with DTIC, vindesine and IFN-α. The patient was vaccinated with 300 μg of peptide, starting in October 1995. At that time, she had about 40 small cutaneous in-transit metastases, the largest one measuring 11 mm diameter. At the time of the third injection, all the cutaneous nodules displayed some flattening. The patient received additional injections of peptide. In the following months, a few cutaneous nodules disappeared and the other lesions were either moderately regressing or stable. In May 1996, small doses of IFN-α were injected in an attempt to favor the putative immune rejection of the tumor by increasing its expression of HLA class I molecules. The patient received $10^6$ U IFN-α s.c., 3 times per week for 4 weeks. No acceleration of the regression process was observed. Vaccination with the MAGE-3 peptide was

FIGURE 2 – Clinical evolution of patients with tumor regression.



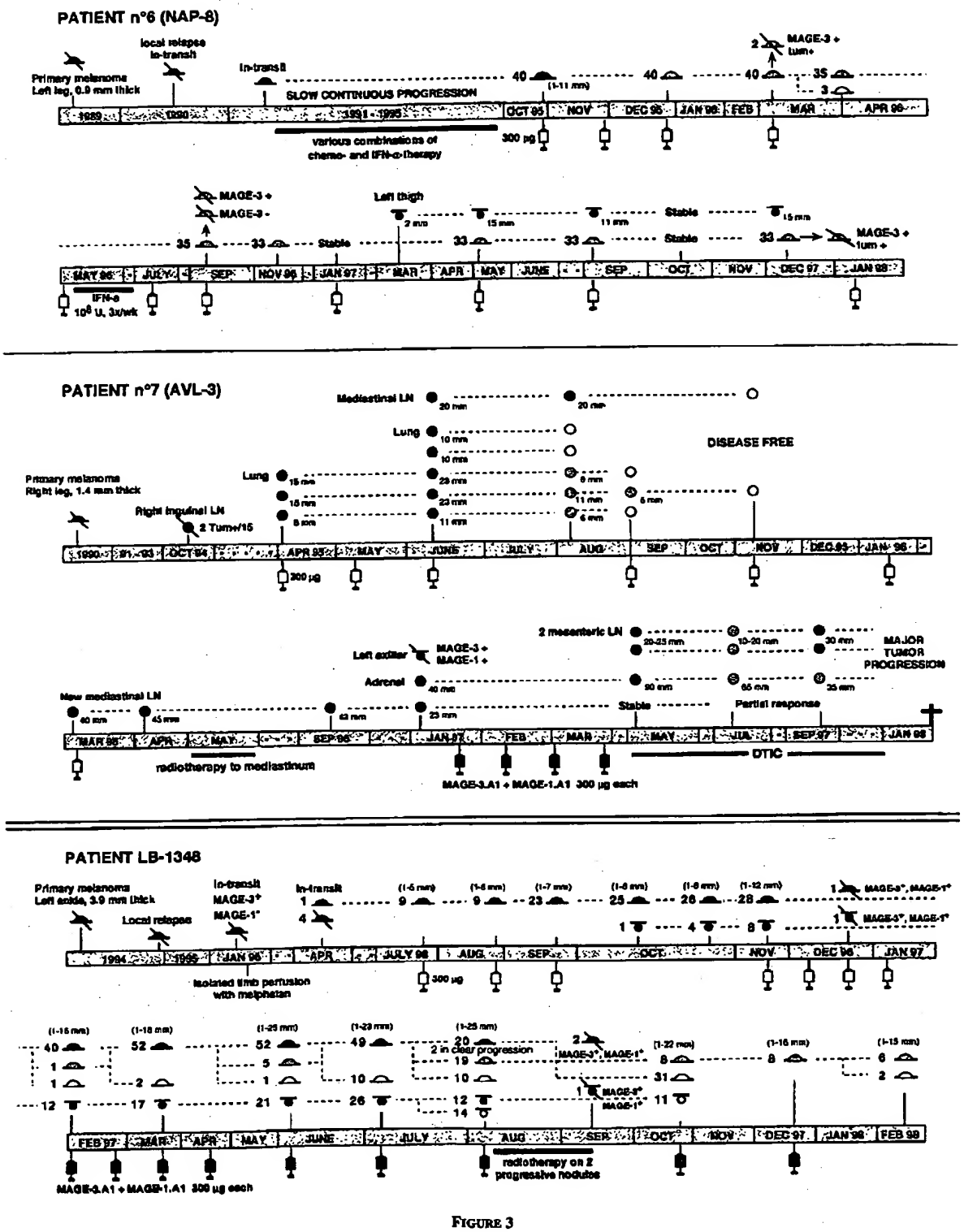

PATIENT n°6 (NAP-8)
Primary melanoma
Left leg, 0.9 mm thick
local relapse
In-transit
SLOW CONTINUOUS PROGRESSION
various combinations of chemo- and IFN-α therapy
MAGE-3 +
MAGE-3 -
Left thigh
Stable
IFN-α
10⁶ U, 3x/wk
PATIENT n°7 (AVL-3)
Mediastinal LN
Lung
DISEASE FREE
Primary melanoma
Right leg, 1.4 mm thick
Right inguinal LN
2 Turn+/15
Left axilla
MAGE-3 +
MAGE-1 +
Adrenal
2 mesenteric LN
MAJOR TUMOR PROGRESSION
New mediastinal LN
Stable
Partial response
radiotherapy to mediastinum
DTIC
MAGE-3.A1 + MAGE-1.A1 300 µg each
PATIENT LB-1348
Primary melanoma
Left axilla, 3.9 mm thick
Local relapse
In-transit
MAGE-3+
MAGE-1+
isolated limb perfusion with melphalan
2 in clear progression
radiotherapy on 2 progressive nodules
MAGE-3.A1 + MAGE-1.A1 300 µg each

FIGURE 3

pursued. Between May 1996 and December 1997, the cutaneous metastases continued to regress slowly, but significantly. However, a small s.c. nodule appeared on the left thigh in March 1997.

In addition to the melanoma lesions of the leg, this patient displayed a suspect lung nodule of 15 mm diameter at the onset of treatment. This nodule remained stable throughout 1996, but increased gradually in size throughout 1997 to reach 25 mm diameter. In February 1998, it was excised and diagnosed as a peripheral lung adenocarcinoma. This second tumor was negative for the expression of the *MAGE-3* gene.

Patient 7 (AVL-3, female, 50 years) had undergone removal of regional lymph node metastases in October 1994 (Fig. 3). She had not received any form of treatment other than surgery. In April 1995, she presented with 3 lung metastases and vaccination with 300 µg of peptide was started. At the time of the third injection, a CT scan revealed an increase in the size of the known lung nodules and 2 new lung metastases. In addition, an enlarged mediastinal lymph node was visible. Two months later, 2 of the 5 lung nodules had disappeared and the 3 remaining nodules had shrunk significantly. The mediastinal lymph node was stable. Because of this favorable evolution, the patient received an additional peptide injection, and no other form of treatment was given. One month later, 1 of the 3 metastases had regressed further and the 2 others had disappeared. Two months later, in November 1995, all the metastatic nodules had disappeared and the mediastinal lymph node had returned to normal size (Fig. 5). The patient displayed no other evidence of disease. She received 3 additional injections with the same amount of peptide, at 2-month intervals, and remained free of disease for an additional 4 months. In March 1996, a new metastatic mediastinal lymph node was observed. Injections were discontinued and local radiotherapy was given to this lesion, which partially regressed and then remained stable. Ten months later, a s.c. and an adrenal metastasis appeared. Because the s.c. metastasis expressed both the *MAGE-3* and *MAGE-1* genes, the patient was included in another study involving vaccination with both the MAGE-3.A1 peptide and a *MAGE-1*-encoded peptide that is also presented by HLA-A1 (Traversari *et al.*, 1992). However, the tumor continued to progress during vaccination. Subsequent chemotherapy with DTIC caused a transient partial response. The patient died from tumor progression, 33 months after entering the treatment with the MAGE-3.A1 peptide. It is noteworthy that during the 22 months following her first relapse the patient acquired progressive lesions in several sites but none in the 5 lung sites or in the mediastinal site that had regressed, suggesting that the regressions at these sites had removed all the tumoral cells, although smaller metastases had escaped the process that caused the regressions.

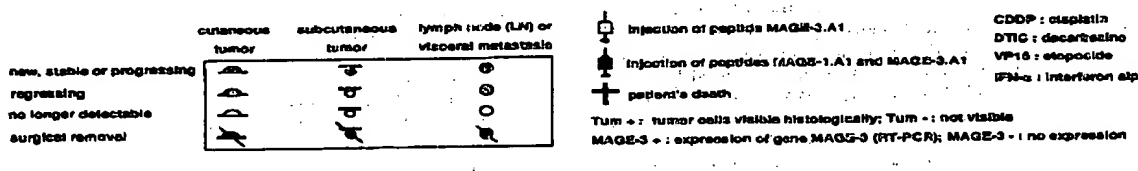
Some features of the regressions that were observed deserve to be mentioned. In some patients, the regressions appeared to have started at a late time: the first signs of regression were observed 4 months after the third immunization in patient 1 and 2 months in patient 7. Late occurrence was not the mark of weak regressions as both patients displayed complete regression. Moreover, once they had started, most regressions proceeded very slowly. It took 9 months for the regressions of patients 1 and 4 to become complete. Patient 6 constitutes a remarkable case with cutaneous lesions that were still decreasing very slowly but continuously 22 months after the first signs of regression were noticed.

In patients 1, 4 and 6, all the cutaneous lesions, which were spread over a wide area, regressed more or less concurrently. However, in patients 2, 3 and 5, the regressions were not homogeneous: some lesions disappeared completely, while others remained stable or progressed.

The occurrence of the slow and incomplete regressions as seen with patients 2, 5 and 6, and that of the relapse after complete regression observed with patient 7, raises the issue of whether the maintenance of a more frequent injection schedule pursued even after complete regression might not have improved the fate of some of these patients. Possibly relevant to this issue are the following observations made with 1 patient who did not regress after immunization with the MAGE-3.A1 peptide. Patient LB-1348 (female, 73 years) had a primary melanoma of the left leg, and a local relapsed lesion removed in 1994 (Figs. 3, 4). A new in-transit metastasis was excised in January 1996 and found to express *MAGE-3* and not *MAGE-1*. Although the patient was treated by limb perfusion with melphalan, new cutaneous in-transit nodules appeared 3 months later. In July 1996, the patient displayed 9 small cutaneous nodules on the left leg, and vaccination with 300 µg of peptide was initiated. By November, the number of the nodules had increased to 28, their size had increased and s.c. lesions had appeared. But a few nodules displayed a necrotic aspect and to favor this putative regressive process, 4 additional injections were given at 2-week intervals. By February 1997, 40 cutaneous and 12 s.c. nodules were spread over the left leg. However, 1 small nodule had become completely flat, while an adjacent 1 showed clear signs of regression. Because a cutaneous nodule removed in December 1996 had been found to express not only *MAGE-3* but also *MAGE-1*, unlike the nodule tested in January 1996, the patient was included in the study involving immunization with both the MAGE-3.A1 and the MAGE-1.A1 peptides. Between February and April 1997, a few additional small cutaneous nodules became completely flat but new cutaneous and s.c. nodules appeared and others enlarged (Fig. 4*c*). The treatment was pursued. By August 1997, there was widespread regression: many cutaneous and s.c. nodules had completely disappeared, while many others had become flatter. However, 2 metastases necessitated local radiotherapy because of progression and ulceration. Following electron beam irradiation limited to an area surrounding the 2 lesions (Fig. 4*c*), both lesions flattened and became necrotic. During the following months, many cutaneous and s.c. nodules located far away from the irradiated region regressed. Additional immunizations were performed. By February 1998, almost all the cutaneous nodules had completely regressed and the others were clearly regressing (Fig. 4*c*). All the s.c. nodules had completely disappeared. No distant metastases were detected. We cannot exclude that the combination of 2 peptides produced a regression that the MAGE-3.A1 peptide would not have produced alone. It is also possible that a similar result could have been achieved by pursuing frequent injections with the single MAGE-3.A1 peptide throughout 1997.

Our results provide no significant evidence that the higher dose of peptide was more effective in inducing regressions than the lower dose. The lower dose was administered to ½ patients who showed tumor regression, to ¼ who were removed from the trial and to ⅛ who showed continuous tumor progression.

FIGURE 3 – Clinical evolution of patients with tumor regression (followed).



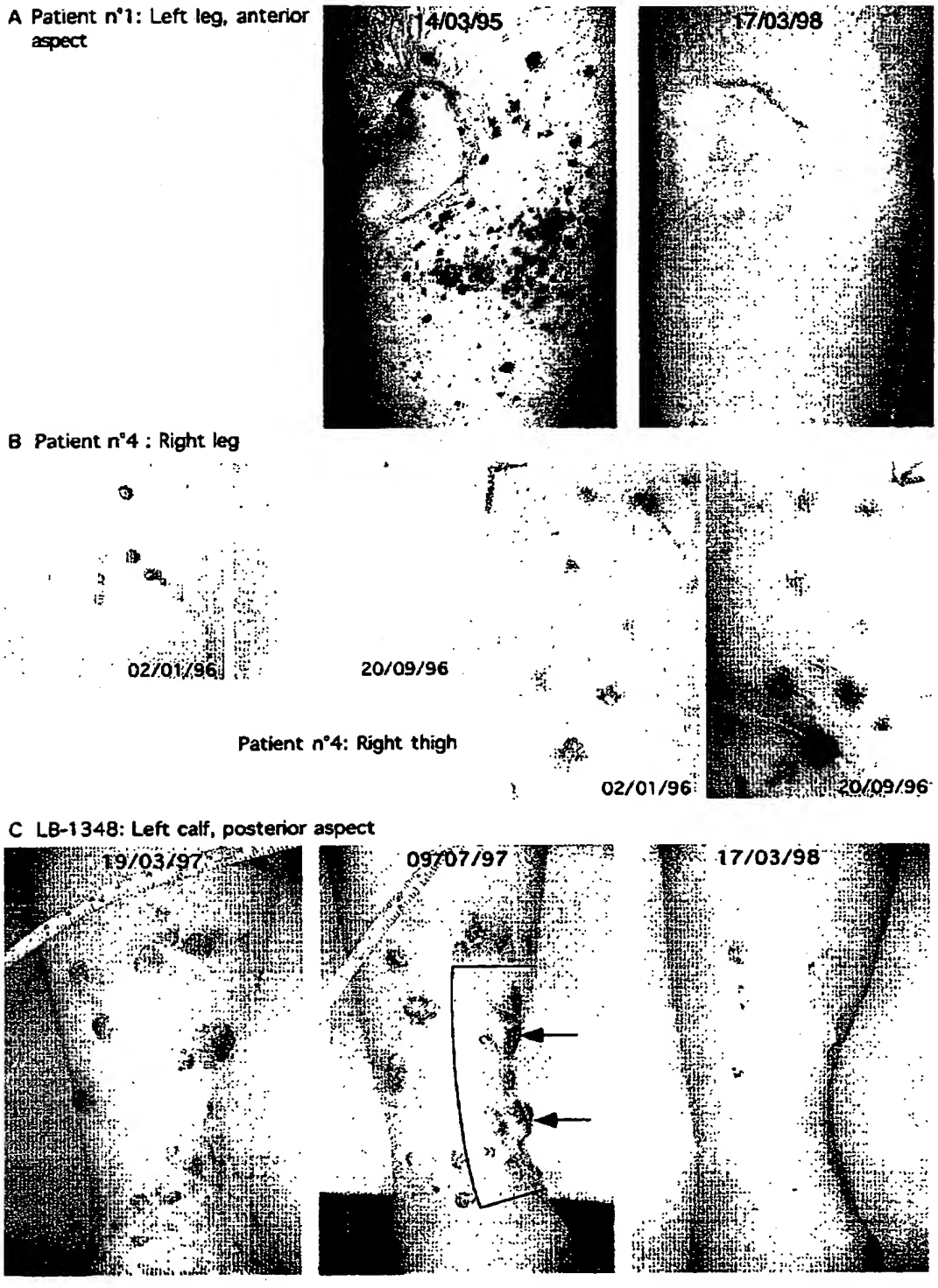



FIGURE 4 – Evolution of cutaneous and subcutaneous in-transit melanoma metastases. For patient LB-1348, the 2 nodules that were irradiated are indicated by arrows; the irradiated field is surrounded by the black line.

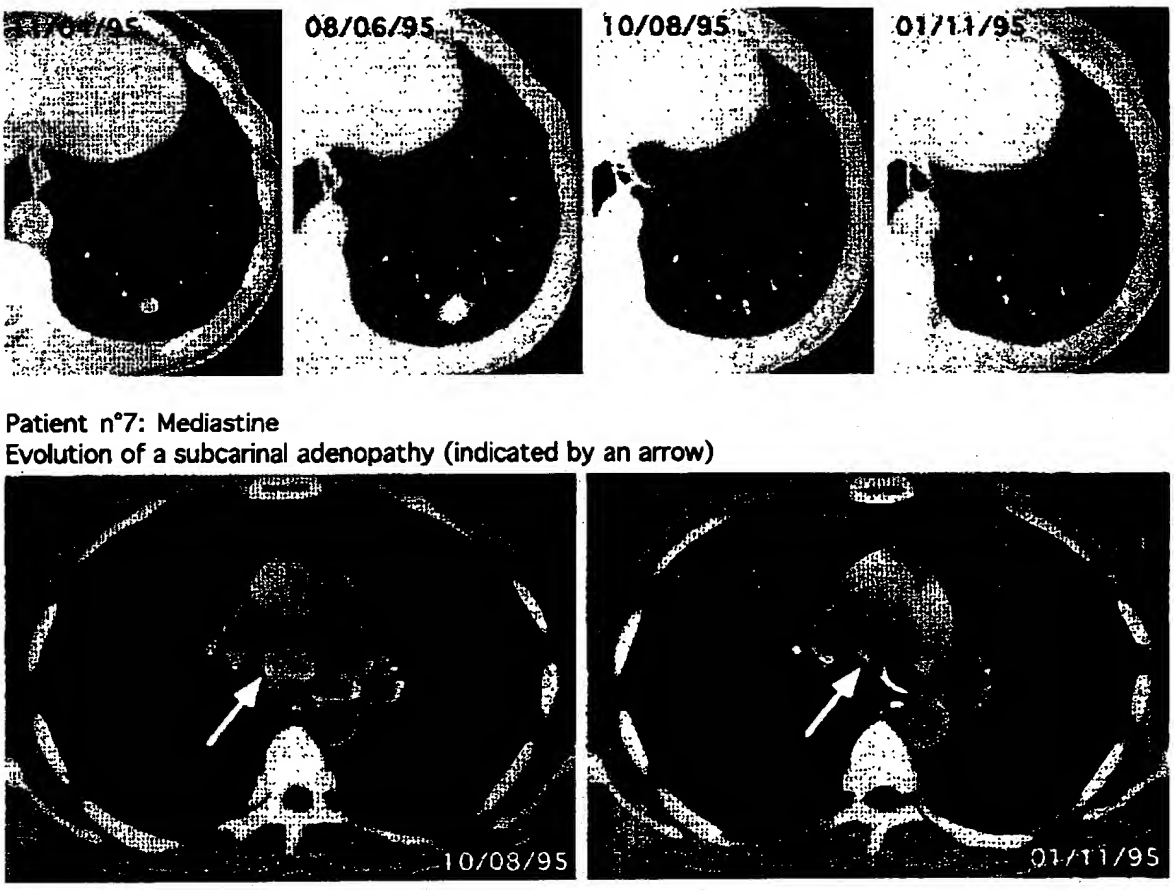



FIGURE 5 – Evolution of distant melanoma metastases.

*Analysis of the CTL response*

We analyzed $5 \times 10^5$ $CD8^+$ T lymphocytes in a blood sample collected from patient 1 (LB-945) during the tumor regression (March 1995). These lymphocytes were stimulated 3 times *in vitro* with cells pulsed with the MAGE-3.A1 peptide (see Method A in the Subjects and Methods section). No CTL activity was observed against targets expressing the MAGE-3.A1 antigen. A similar analysis was performed with $10^6$ lymphocytes from patient 7 (AVL-3) collected when regression was ongoing (August 1995) or complete (January 1996). The results were also negative. The lymphocytes of patients 1 and 7 produced significant CTL activity upon stimulation with an influenza peptide, indicating that the lymphocytes of these patients remained capable of responding to antigenic stimulation. The lymphocytes of patients LB-1045 and LB-278, for whom neither regression nor stabilization was observed, were also restimulated with the MAGE-3.A1 peptide and no CTL activity specific for targets expressing the MAGE-3.A1 antigen was detected.

Stimulation of large numbers of $CD8^+$ T lymphocytes (more than $10^7$) by peptide-pulsed cells has made it possible to evaluate the frequency of anti-MAGE-3.A1 CTL precursors in cancer-free individuals (Chaux *et al.*, 1998). This frequency was estimated at $2 \times 10^{-7}$. The presence of anti-MAGE-3.A1 CTL precursors was tested using this method in $2 \times 10^7$ blood $CD8^+$ T lymphocytes of patient 7, 2 months after documentation of a complete regression (see Method B in the Subjects and Methods section). The observed frequency was $1.3 \times 10^{-7}$.

Thus, the complete regressions observed in patients 1 and 7 were not paralleled by a strong increase in circulating CTL precursors directed against the MAGE-3.A1 antigen.

## DISCUSSION

Of a total of 39 melanoma tumor-bearing patients who received the MAGE-3.A1 peptide, 14 were removed after only 1 or 2 injections because of fast progression, and 18 showed continuous progression without any regression or significant stabilization, but 7 patients showed significant regressions. Three regressions were complete and 2 of these have led to a disease-free state maintained for more than 2 years after the beginning of treatment. These regressions are very likely to be linked to the treatment, given the very low frequency of spontaneous complete regressions reported for metastatic melanoma, i.e. 0.4% (Baldo *et al.*, 1991).

Fourteen patients were withdrawn after 1 or 2 injections because other treatments were deemed necessary to cope with the progression of the disease. Most of these patients had distant metastases

and had failed to respond to multiple previous treatments. Because the clinical course of melanoma is unpredictable, it is impossible to exclude that our treatment accelerated the course of the disease in some patients, but we feel that there is no significant evidence to support this conclusion.

Should we consider that the patients withdrawn from the study have failed to respond to the treatment? Insofar as all but 1 patient who displayed regressions did so only from the time of the third injection or later, it is tempting to consider that early withdrawal prevented the observation of the full potential of the treatment. This is, however, a secondary issue, as the aim of our limited study was not to evaluate the efficacy with any degree of precision, but rather to find out whether regressions could be observed. Interestingly, the proportion of withdrawn patients decreased as the study proceeded, as it was realized that regressions occurred late in the process.

Although the regressions appear to be linked to the treatment, we have failed to obtain any evidence supporting the anticipated mechanism of anti-tumor response, namely the development of a strong anti-MAGE-3 CTL response capable of destroying the tumor cells. No evidence was found for any significant increase of anti-MAGE-3 CTL precursors in the blood following repeated immunizations. Nevertheless, it is possible that a weak CTL response was produced in the regressor patients, but that these CTLs migrated immediately to the vicinity of the tumor so that they could not be found in the blood. In agreement with this notion, a T-cell receptor (TCR) repertoire study performed on a lesion excised after immunization of patient 3, who showed cutaneous regressions, indicated that expansion of a T-cell clone, expressing TCRBV20/TCRAV27, occurred. This T-cell clone was also found, but at low frequency, in a different pre-vaccination metastasis but not in peripheral blood lymphocytes either before or after vaccination (data not shown). It is also possible that the putative responder T lymphocytes were located almost exclusively in lymph nodes. Nevertheless, we believe that a massive CTL response would have been observed with our tests. Our preferred interpretation is that the peptide injections produced a weak CTL response in some of the patients, including the patients in whom tumor regressions were observed. This interpretation is compatible with the slow pace of the tumor regressions.

If one accepts the tentative conclusion that the regressions are linked to the immunizations, it is worth considering why about 30% of the patients who received the full treatment showed significant regressions whereas 70% did not show any regression. Among the possible factors, we will consider the extent of the disease, the amplitude of the T lymphocyte response to the vaccine and various properties of the tumor cells.

Our limited data do not demonstrate a strict correlation between the stage of the disease and the occurrence of regressions. However, as might have been expected, there is a definite trend toward better results with less advanced forms of disease. Six of the 7 regressions occurred in cutaneous metastases, 5 regional and 1 distant. Cutaneous melanoma metastases are also those which respond better to chemotherapy, radiotherapy and cytokine treatment than more advanced metastases (Balch, 1992).

It is clear that the tumor mass present at the onset of treatment does not determine the outcome. In addition to the tumor-bearing melanoma patients, some of whom had regressions, 6 patients who had been rendered tumor-free by surgery received the vaccine. Yet they all relapsed. The set of patients, who had only in-transit cutaneous metastases, will probably be most suitable for the study of the other factors that influence the outcome of the treatment, as some of these patients displayed complete and long-term regressions, whereas others failed to show any regression.

A determining factor may have been the intensity of the immune response to the vaccine. Our results could be explained by the occurrence of a modest immune response in the regressing patients and a complete lack of response in the others. This finding could be the result of a stochastic process whereby each peptide injection would have a probability of, say, 10% of triggering a CTL response. After 3 immunizations, about one-third of the patients would have initiated a response, and all of these CTL responses would have produced noticeable regressions. If this interpretation is correct, it follows that increasing the number and the frequency of injections ought to improve the results. A clinical study based on this hypothesis has been initiated. On the other hand, the various patients may differ in their ability to be immunized, due for instance to different frequencies of anti-MAGE-3.A1 CTL precursors. Possibly, the patients with a lower response potential require better modes of immunizations, such as MAGE protein combined with adjuvant. This procedure is presently being tested in a clinical study involving the MAGE-3 protein. Defective recombinant poxviruses or adenoviruses carrying *MAGE* sequence may also be more effective immunogens than peptide. A recombinant adenovirus has been found to be effective in producing CTL responses in mice against tumor antigen P815AB (Warnier *et al.*, 1996).

In addition to the difficulty in eliciting anti-tumor immune response, it is possible that even when these responses can be induced, they remain weak and rapidly subside, so that even in those patients in whom tumor regression is initiated, vaccination must be pursued to boost the immune response and render it capable of bringing the regression to completion. The observation made with patient LB-1348 suggests that results might be improved by numerous and frequent vaccinations. Likewise, those patients who display a complete regression, may have to be immunized regularly for several years to maintain a sufficient level of immune protection against emerging metastases.

Many properties of the tumor cells could affect the outcome of treatment. A first set relates directly to the presence of the antigen. Tumor cells vary considerably in their extent of expression of the *MAGE-3* gene. They can also vary in their general ability to present antigens on class I MHC molecules. This variability depends on the level of expression of HLA genes and on the integrity of the antigen-processing mechanism, which involves the Transporter associated with antigen processing (TAP) transporter proteins and proteasomes. These properties are known to differ between tumors of different individuals and between individual metastases of the same patient. They may even be the object of microheterogeneity within the same metastasis. Rare cells having lost the antigen could be selected by the anti-tumoral process and cause subsequent relapse.

The level of *MAGE-3* expression measured in a tumor sample prior to treatment was not clearly correlated with the occurrence of regressions. The tumor samples of regressing patients NAP-10 and LB-1448 had only low levels of MAGE-3 mRNA. But the degree of expression of *MAGE* genes in tumor samples is subject to underestimation due to the presence of contaminating normal cells. Conversely, high expression was not sufficient to ensure success. Several patients, including LB-1045 and NAP-13, who had only regional disease, had tumors containing very high levels of MAGE-3 mRNA and nevertheless did not show any sign of regression.

Heterogeneity of *MAGE* expression among different metastases might explain why some metastases regressed while others remained stable, progressed, or emerged, as seen in patients LB-1448, MI-28, KI-1, NAP-8 and AVL-3. Unfortunately, it has not been possible to analyze *MAGE-3* expression of most of these metastases. As seen with patients LB-1448 and AVL-3, the progressing lesions were often *MAGE-3* positive. In patient KI-1, 2 progressing lesions did not express *MAGE-3*, and the third was *MAGE-3* positive. We conclude that failure of some metastases to express *MAGE-3* is not the sole explanation for the heterogeneity in the regression process. A new metastasis, which occurred and progressed in patient AVL-3 after her complete response, was *MAGE-3* positive. This new metastasis was cultured and found to be lysed by anti-MAGE-3 CTL, indicating that this progression could not be explained by failure to present the antigen.

Local escape by selection of rare cells that fail to express the MAGE-3.A1 antigen has not been noticed, because we have not seen any recurrence on sites that had completely regressed. This observation was particularly striking with patient AVL-3, whose lungs remained clear even when she had numerous progressing visceral metastases in other locations. Perhaps escape will not appear as a critical issue until such time as long-term complete regression of large tumor masses are obtained.

Other properties than those affecting the presence of the antigen on the tumor might affect the outcome of therapeutic vaccination. Some relate to the accessibility of the tumor to the CTL. Tumors have been reported to be fed by microvessels with an abnormal endothelium, which prevents proper migration of T lymphocytes into the tumor (Jain *et al.*, 1996; Piali *et al.*, 1995). This abnormal endothelium might explain why in some patients some lesions regress completely while others progress: the initial access of lymphocytes to a lesion would be a rare occurrence, but once a small number of CTL had entered a lesion, they could initiate a positive feedback process attracting a large number of other CTLs, leading to the complete regression of that lesion. Once T lymphocytes have gained access to the tumor, their activation may be inhibited by tumor growth factor (TGF)-β, or they could be killed by FAS ligands expressed by the tumor cells (Hahne *et al.*, 1996; Inge *et al.*, 1992). Finally, it is possible that the individual makeup of the tumors, including oncogenes and anti-oncogenes, might affect their sensitivity to immunotherapy.

A major limitation of the effectiveness of the anti-tumor rejection process could be that the CTL elicited by the MAGE-3.A1 peptides that reach the tumor fail to be restimulated by the tumor cells, so that massive proliferation and activation does not occur at the tumor site. A similar situation has been observed in a transgenic mouse model, in which a tumor carrying a lymphocytic choriomeningitis virus (LCMV) antigen not only failed to induce a CTL response against this antigen, but also failed to maintain a response induced with LCMV (Speiser *et al.*, 1997). Perhaps only simultaneous vaccination against tumor-specific antigens presented by class I and class II HLA molecules will improve this situation: as a few $CD8^+$ CTL would start destroying a few tumor cells in a lesion, tumor-specific proteins of these cells could be taken up and presented to $CD4^+$ T helper cells that have been stimulated by the class II antigens of the vaccine. These T helper cells could then secrete cytokines that would co-stimulate the CTL and produce a positive feedback loop leading to fast and complete elimination of all the tumor cells. A class II determinant has recently been identified for MAGE-3 (data not shown).

Several tumor regressions have been observed in melanoma patients immunized with antigenic peptides, dendritic cells pulsed with antigenic peptides or antigenic peptides combined with high-dosage IL-2 (Jaeger *et al.*, 1996; Nestle *et al.*, 1998; Rosenberg *et al.*, 1998). We are increasingly convinced that immunization with tumor-specific defined antigens can produce some tumor regressions, and that this regression occurs in the absence of any toxicity. Considerable further progress is needed, however, before immunization with tumor-specific antigens recognized by T cells becomes an effective and generally applicable cancer therapy. Our results provide some orientation for future work and suggest that success may be achievable.

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REVIEW

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# A listing of human tumor antigens recognized by T cells

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**Key words** Antigens · Tumor · T cells · Epitopes

**Complete list of abbreviations of tumor antigens** *707-AP* 707 alanine proline · AFP *alpha* (α)*-fetoprotein* · *ART-4* adenocarcinoma antigen recognized by T cells 4 · *BAGE* B antigen · *β-catenin/m* β-catenin/mutated · *Bcr-abl* breakpoint cluster region–Abelson · *CAMEL* CTL-recognized antigen on melanoma · *CAP-1* carcinoembryonic antigen peptide-1 · *CASP-8* caspase-8 · *CDC27m* cell-division-cycle 27 mutated · *CDK4/m* cycline-dependent kinase 4 mutated · *CEA* carcinoembryonic antigen · *CT* cancer/testis (antigen) · *Cyp-B* cyclophilin B · *DAM* differentiation antigen melanoma (the epitopes of DAM-6 and DAM-10 are equivalent, but the gene sequences are different; DAM-6 is also called MAGE-B2, and DAM-10 is also called MAGE-B1) · *ELF2M* elongation factor 2 mutated · *ETV6-AML1* Ets variant gene 6/acute myeloid leukemia 1 gene ETS · *G250* glycoprotein 250 · *GAGE* G antigen · *GnT-V* N-acetylglucosaminyltransferase V · *Gp100* glycoprotein 100 kDa · *HAGE* helicose antigen · *HER-2/neu* human epidermal receptor-2/neurological · *HLA-A*0201-R170I* arginine (R) to isoleucine (I) exchange at residue 170 of the α-helix of the α2-domain in the HLA-A2 gene · *HPV-E7* human papilloma virus E7 · *HSP70-2M* heat shock protein 70-2 mutated · *HST-2* human signet ring tumor-2 · *hTERT* or *hTRT* human telomerase reverse transcriptase · *iCE* intestinal carboxyl esterase · *KIAA0205* name of the gene as it appears in databases · *LAGE* L antigen · *LDLR/FUT* low-density lipid receptor/GDP-L-fucose: β-D-galactosidase 2-α-L-fucosyltransferase · *MAGE* melanoma antigen · *MART-1/Melan-A* melanoma antigen recognized by T cells-1/melanoma antigen A · *MC1R* melanocortin 1 receptor · *Myosin/m* myosin mutated · *MUC1* mucin 1 · *MUM-1, -2, -3* melanoma ubiquitous mutated 1, 2, 3 · *NA88-A* NA cDNA clone of patient M88 · *NY-ESO-1* New York-esophagus 1 · *P15* protein 15 · *p190* minor bcr-abl protein of 190 kDa bcr-abl · *Pml/RARα* promyelocytic leukaemia/retinoic acid receptor α · *PRAME* preferentially expressed antigen of melanoma · *PSA* prostate-specific antigen · *PSM* prostate-specific membrane antigen · *RAGE* renal antigen · *RU1* or *RU2* renal ubiquitous 1 or 2 · *SAGE* sarcoma antigen · *SART-1* or *SART-3* squamous antigen rejecting tumor 1 or 3 · *TEL/AML1* translocation Ets-family leukemia/acute myeloid leukemia 1 · *TPI/m* triosephosphate isomerase mutated · *TRP-1* tyrosinase related protein 1, or gp75 · *TRP-2* tyrosinase related protein 2 · *TRP-2/INT2* TRP-2/intron 2 · *WT1* Wilms' tumor gene

**Abbreviations used** *ALL* acute lymphoblastic leukemia · *AML* acute myeloid leukemia · *APL* acute promyelocytic leukemia · *CML* chronic myelogenous leukemia · *CTL* cytotoxic T lymphocytes · *Ets* E-26 transforming specific (family of transcription factors) · *H/N* head and neck · *MHC* major histocompatibility complex · *NSCLC* non-small cell lung carcinoma · *ORF* open reading frame · *RCC* renal cell carcinoma · *SCC* squamous cell carcinoma · *TSTA* tumor-specific transplantation antigens

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## Introduction

Since the cloning of *MAGE-1* [125], the first gene reported to encode a human tumor antigen recognized by T cells, molecular identification and characterization of tumor antigens has mainly been achieved for melanoma. A major reason for this lies in the difficulty of establishing cell lines in vitro from other types of cancer, such lines being necessary to generate tumor-specific CTL

lines or clones to be used in the genetic or biochemical approach aimed at molecularly identifying new cancer antigens. More recently, however, new approaches have allowed the discovery of new antigens recognized by T cells even in tumors other than melanoma.

It is, then, important to categorize these antigens, particularly for the HLA allele restricting their recognition by T cells and for their tissue distribution. With this purpose, tumor antigens have been collected in the present work and briefly commented.

The list presented in the tables below includes all T-cell-defined epitopes encoded by tumor antigens and published by 31 July 2000. Analogs or artificially modified epitopes are excluded from the list. Only tumor antigens recognized by T cells (either cytotoxic CD8+ or helper CD4+) are listed, given their potential importance in the control of tumor growth. Other antigens, identified by antibodies, are excluded but a large collection of them, as detected by the Serex technology, can be found in the data base of the Institute for Cancer Research (www.licr.org/SEREX.htm). It is of note that many tumor antigens (e.g. MAGE, NY-ESO-1a) are now known to be recognized by both T cells and antibodies in the same cancer patients [54].

In the tables herein, tumor antigens are listed in alphabetic order along with the epitope sequence and the HLA allele which restricts recognition by T cells. Furthermore, data on the tissue distribution of each antigen are provided, making this listing an important source for easily retrieving data concerning human tumor antigens.

The listing is meant to be a tool for scientists and students who have an interest in the field of tumor immunology and immunotherapy. The bibliography allows a rapid search for more detailed information at the single antigen or epitope level.

We do not ignore, however, the fact that by recent technologies (e.g., subtractive hybridization, representational-difference analysis, microarrays) hundreds of genes are being detected which are preferentially expressed or overexpressed in neoplastic cells as compared with normal counterparts or are expressed in metastatic but not in primary, early lesions (e.g., melanoma, breast cancer, lymphoma). By using appropriate computer algorithms [9], a number of new epitopes will be identified that can bind MHC molecules. By applying such approaches, a large array of gene products can be screened for their potential antigenic function. More cumbersome may be the selection of the most immunogenic epitopes through appropriate functional assays.

## Classification of tumor antigens

### Group 1: Class I HLA-restricted cancer/testis antigens (Table 1)

A milestone in tumor immunology was certainly the cloning of *MAGE-1* [125] and the subsequent characterization of the first T-cell-defined antigenic epitope a year later [119]. Those findings were rapidly followed by the identification of new members within this group [6, 123]. The *MAGE*, *BAGE* and *GAGE* families of genes were born. The antigens belonging to this group, now including also NY-ESO-1, were called cancer/testis (CT) antigens for their expression in histologically different human tumors and, among normal tissues, in spermatocytes/spermatogonia of testis and, occasionally, in placenta. These antigens now represent one of the main components for antitumor vaccine development. CT antigens result from reactivation of genes normally silent in adult tissues [27], but that are transcriptionally activated in some tumors [30]. Their expression in testis does not provide targets for an immune reaction because cells of testis do not express class I HLA [56]. Despite the fact that the CT genes are probably the most characterized ones, their physiological function remains largely unknown.

Considering that new genes in the group of CT antigens have been cloned (CT9 [105], CT10 [46], LAGE [72], MAGE-B5, –B6, –C2, –C3 and –D [74, 75], HAGE, SAGE [80]), but that no T-cell epitopes have been identified from them yet, the question arises as to how many more genes encoding CT antigens remain to be discovered and how many epitopes exist that could be of use in cancer immunotherapy.

### Group 2: Class I HLA-restricted differentiation antigens (Table 2)

These antigens are shared between tumors and the normal tissue from which the tumor arose; most are found in melanomas and normal melanocytes [2]. Many of these melanocyte lineage-related proteins are involved in the biosynthesis of melanin. Epitopes recognized by both CD8+ and CD4+ T cells can be derived from melanosome proteins [8, 118, 135, 136].

### Group 3: Class I HLA-restricted widely expressed antigens (Table 3)

Genes encoding widely expressed tumor antigens have been detected in many normal tissues as well as in histologically different types of tumors with no preferential expression on a certain type of cancer. It is possible that the many epitopes expressed on normal tissues are below the threshold level for T-cell recognition, while their overexpression in tumor cells can trigger an anticancer response even by breaking a previously established tolerance. These widely expressed gene products have revealed a broad spectrum of mechanisms that are involved in generating T-cell-defined epitopes through alterations in gene transcription and translation. To highlight some examples, the epitope of CEA is derived from a non-AUG-defined alternative ORF [1], while the RU2 gene creates its epitope by reverse strand transcription [124].

Table 1 Class I HLA-restricted cancer/testis antigens. All these antigens were found to be expressed by normal spermatocytes and/or spermatogonia of testis. Occasionally *MAGE-3*, *MAGE-4* and the *GAGE* genes were found to be expressed also in placenta [26, 24]. The NY-ESO-1 antigen was found to be expressed in normal ovary cells [18]

| Gene | HLA allele | Peptide epitope | Authors [ref.] | Tissue distribution among tumors[a] |
|---|---|---|---|---|
| *MAGE-A1* | A1 | EADPTGHSY | Traversari et al. 1992 [119] | Melanoma, breast carcinoma, SCLC [27, 29, 125] – sarcoma, NSCLC [27, 29] – thyroid medullary carcinoma [125] – colon carcinoma [27] – laryngeal tumors [29] |
| *MAGE-A1* | A3 | SLFRAVITK | Chaux et al. 1999a [16] | |
| *MAGE-A1* | A24 | NYKHCFPEI | Fujie et al. 1999 [37] | |
| *MAGE-A1* | A28 | EVYDGREHSA | Chaux et al. 1999a [16] | |
| *MAGE-A1, -A2, -A3, -A6* | B37 | REPVTKAEML | Tanzarella et al. 1999 [113] | Melanoma, colon and breast carcinomas, SCLC [27, 29, 125] – sarcoma, NSCLC [27, 29] – thyroid medullary carcinoma, H/N tumors, bronchial SCC [125] – laryngeal tumors [29] – leukemias [27] |
| *MAGE-A1* | B53 | DPARYEFLW | Chaux et al. 1999a [16] | Melanoma, breast carcinoma, SCLC [27, 29, 124] – sarcoma, colon carcinoma, NSCLC [27, 29] – thyroid medullary carcinoma [125] |
| *MAGE-A1* | Cw2 | SAFPTTINF | Chaux et al. 1999a [16] | |
| *MAGE-A1* | Cw3 | SAYGEPRKL | Chaux et al. 1999a [16] | |
| *MAGE-A1* | Cw16 | SAYGEPRKL | van der Bruggen et al. 1994b [127] | |
| *MAGE-A2* | A2 | KMVELVHFL | Visseren et al. 1997 [128] | Melanoma, colon and breast carcinomas, SCLC [27, 29, 125] – sarcoma, NSCLC [27, 29] – thyroid medullary carcinoma [125] – laryngeal tumors [77] – leukemias [27] |
| *MAGE-A2* | A2 | YLQLVFGIEV | Visseren et al. 1997 [128] | |
| *MAGE-A2* | A24 | EYLQLVFGI | Tahara et al. 1999 [110] | |
| *MAGE-A3* | A1 | EADPIGHLY | Gaugler et al. 1994 [40] | Melanoma, colon and breast carcinomas [27, 125] – H/N tumors [18] – bronchial SCC, thyroid medullary and bladder carcinoma, sarcomas, SCLC, NSCLC [125] – leukemias [29] |
| *MAGE-A3* | A2 | FLWGPRALV | van der Bruggen et al. 1994a [126] | |
| *MAGE-A3* | A24 | TFPDLESEF | Oiso et al. 1999 [89] | |
| *MAGE-A3* | A24 | IMPKAGLLI | Tanaka et al. 1997 [111] | |
| *MAGE-A3* | B44 | MEVDPIGHLY | Herman et al. 1996 [48], Fleischhauer et al. 1996 [35] | |
| *MAGE-A3* | B52 | WQYFFPVIF | Russo et al. 2000 [103] | |
| *MAGE-A4* | A2 | GVYDGREHTV | Duffour et al. 1999 [33] | Melanoma, NSCLC, sarcomas, esophageal, colon and breast carcinomas [27] |
| *MAGE-A6* | A34 | MVKISGGPR | Zorn and Hercend, 1999b [147] | Melanoma, NSCLC, colon carcinoma, leukemias [27] |
| *MAGE-A10* | A2 | GLYDGMEHL | Huang et al. 1999 [52] | Not defined |
| *MAGE-A12* | Cw7 | VRIGHLYIL | Panelli et al. 2000 [91], Heidecker et al. 2000 [47] | Melanoma, myeloma, brain tumors, sarcoma, leukemias, SCLC, NSCLC, H/N tumors, bladder, lung, esophageal, breast, prostate and colorectal carcinoma [27] |
| *BAGE* | Cw16 | AARAVFLAL | Boël et al. 1995 [6] | Melanoma, bladder and mammary carcinomas, H/N SCC, NSCLC, sarcoma |
| *DAM-6, -10* | A2 | FLWGPRAYA | Fleischhauer et al. 1998 [36] | Melanoma, skin tumors, mammary and ovarian carcinomas [77] – lung carcinoma [25, 77] – seminomas [25] |
| *GAGE-1, -2, -8* | Cw6 | YRPRPRRY | Van den Eynde et al. 1995 [123], De Backer et al. 1999 [26] | Melanoma, sarcoma, NSCLC, SCLC, mesothelioma, sarcoma, seminoma, leukemias, lymphomas, H/N tumors, bladder, esophageal, mammary, colon, prostate carcinomas |
| *GAGE-3, -4, -5, -6, -7B* | A29 | YYWPRPRRY | De Backer et al. 1999 [26] | Melanomas, H/N tumors, leukemias, esophageal, lung and bladder carcinomas |
| *NA88-A* | B13 | MTQGQHFLQKV | Moreau-Aubry et al. 2000 [82] | Melanoma |
| *NY-ESO-1* | A2 | SLLMWITQCFL | Jäger et al. 1998 [54] | Melanoma, sarcoma, B-lymphomas, hepatoma, H/N tumors, bladder, lung, prostate, ovarian, thyroid and breast carcinoma [18] |
| *NY-ESO-1a (CAG-3)* | A2 | SLLMWITQC | Jäger et al. 1998 [54] | |
| | A2 | QLSLLMWIT | Jäger et al. 1998 [54] | |
| | A31 | ASGPGGGAPR | Wang et al. 1998b [134] | |

[a] Tissue distribution among tumors as described in the given references when different from the paper first reporting the sequence of the epitope

## Group 4: Class I HLA-restricted, tumor-specific antigens (Table 4)

Unique tumor antigens arise from point mutations of n rmal genes (like β-catenin, CDK4) [98, 137], whose molecular changes often accompany neoplastic transformation or progression. These antigens are thus expressed only in the individual tumor where they were identified, since it is unlikely that the same mutation may occur in two different n oplasms unless it involves genes (e.g. RAS) wh se alteration is an obligatory step in neoplastic transformation.

**Table 2** Class I HLA-restricted melanocyte differentiation antigens. These antigens can only be expressed in normal and neoplastic cells of the same lineage (namely melanocytes, skin, retina, peripheral ganglia) or in normal cells of the prostate gland

| Gene | HLA allele | Peptide epitope | Authors [ref.] |
|---|---|---|---|
| *MART-1/Melan-A*[a] | A2 | AAGIGILTV | Coulie et al. 1994 [22], Kawakami et al. 1994a [58] |
| | A2 | EAAGIGILTV | Schneider et al. 1998 [106] |
| | A2 | ILTVILGVL | Castelli et al. 1995 [14] |
| | B45 | AEEAAGIGIL | Schneider et al. 1998 [106] |
| | B45 | AEEAAGIGILT | Schneider et al. 1998 [106] |
| *MC1R* | A2 | TILLGIFFL | Salazar-Onfray et al. 1997 [104] |
| | A2 | FLALIICNA | Salazar-Onfray et al. 1997 [104] |
| *Gp100* | A2 | KTWGQYWQV | Bakker et al. 1995 [3] |
| | A2 | AMLGTHTMEV | Tsai et al. 1997 [120] |
| | A2 | MLGTHTMEV | Tsai et al. 1997 [120] |
| | A2 | SLADTNSLAV | Tsai et al. 1997 [120] |
| | A2 | ITDQVPFSV | Kawakami et al. 1995 [61] |
| | A2 | LLDGTATLRL | Kawakami et al. 1994b [59] |
| | A2 | YLEPGPVTA | Cox et al. 1994 [24] |
| | A2 | VLYRYGSFSV | Kawakami et al. 1995 [61] |
| | A2 | RLMKQDFSV | Kawakami et al. 1998 [62] |
| | A2 | RLPRIFCSC | Kawakami et al. 1998 [62] |
| | A3 | LIYRRRLMK | Kawakami et al. 1998 [62] |
| | A3 | ALNFPGSQK | Kawashima et al. 1998 [65] |
| | A3 | SLIYRRRLMK | Kawashima et al. 1998 [65] |
| | A3 | ALLAVGATK | Skipper et al. 1996 [108] |
| | A24 | VYFFLPDHL | Robbins et al. 1997 [99] |
| | Cw8 | SNDGPTLI | Castelli et al. 1999 [15] |
| *PSA* | A1 | VSHSFPHPLY | Corman et al. 1998 [20] |
| | A2 | FLTPKKLQCV | Correale et al. 1997 [21] |
| | A2 | VISNDVCAQV | Correale et al. 1997 [21] |
| *PSM* | A1 | HSTNGVTRIY | Corman et al. 1998 [20] |
| *Tyrosinase* | A1 | KCDICTDEY | Kittlesen et al. 1998 [68] |
| | A1 | SSDYVIPIGTY | Kawakami et al. 1998 [62] |
| | A2 | YMDGTMSQV | Wölfel et al. 1994 [137] |
| | A2 | MLLAVLYCL | Wölfel et al. 1994 [137] |
| | A24 | AFLPWHRLF | Kang et al. 1995 [57] |
| | B44 | SEIWRDIDF | Brichard et al. 1996 [10] |
| *TRP-1 (or gp75)* | A31 | MSLQRQFLR | Wang et al. 1996b [132] |
| *TRP-2* | A2 | SVYDFFVWL | Parkhurst et al. 1998 [92] |
| | A2 | TLDSQVMSL | Noppen et al. 2000 [86] |
| | A31 | LLGPGRPYR | Wang et al. 1996a [131] |
| | A33 | LLGPGRPYR | Wang et al. 1998a [133] |
| | Cw8 | ANDPIFVVL | Castelli et al. 1999 [15] |

[a] Two different groups simultaneously discovered this gene and gave it two different names, MART-1 and Melan-A respectively

**Table 3** Class I HLA-restricted widely expressed antigens

| Gene | HLA | Peptide epitope | Tissue distribution | | Reference |
|---|---|---|---|---|---|
| | | | Tumors | Normal tissues | |
| *ART-4* | A24 | AFLRHAAL DYPSLSATDI | SCC, SCLC, H/N tumors, leukemia, lung, esophageal, gastric, cervical, endometrial, ovarian and breast carcinomas | Testis, placenta, fetal liver | Kawano et al. 2000 [64] |
| *CAMEL* | A2 | MLMAQEALAFL | Melanoma | Testis, placenta, heart, skeletal muscle, pancreas | Aarnoudse et al. 1999 [1] |
| *CEA* | A2 | YLSGANLNL (CAP-1)[a] | Melanoma | Testis, placenta, heart, skeletal muscle, pancreas | Tsang et al. 1995 [121] |
| *CEA* | A3 | HLFGYSWYK | Colon, rectum, pancreas, gastric, breast and lung carcinomas | Gastrointestinal embryonic tissue | Kawashima et al. 1999 [66] |
| *Cyp-B* | A24 | KFHRVIKDF DFMIQGGDF | Lung adenocarcinoma, T cell leukemia, lymphosarcoma – bladder, ovarian, uterine and esophageal SCC | Ubiquitously expressed in normal tissues | Gomi et al. 1999 [42] |

**Table 3** (Continued)

| Gene | HLA | Peptide epitope | Tissue distribution | | Reference |
|---|---|---|---|---|---|
| | | | Tumors | Normal tissues | |
| *HER2/neu* | A2 | KIFGSLAFL | Melanoma – ovarian and breast carcinomas | Epithelial cells | Fisk et al. 1995 [34] |
| *HER2/neu* | A2 | IISAVVGIL | Melanoma, ovarian, pancreatic [96][b] and breast carcinomas | Epithelial cells | Peoples et al. 1995 [95] |
| *HER2/neu* | A2 | RLLQETELV | Melanoma, ovarian, gastric, pancreatic [96] and breast carcinomas | Epithelial cells | Kono et al. 1998 [71] |
| *HER2/neu* | A2 | VVLGVVFGI ILHNGAYSL YMIMVKCWMI | Melanoma, ovarian, gastric, pancreatic [96] and breast carcinomas | Epithelial cells | Rongcun et al. 1999 [101] |
| *HER2/neu* | A3 | VLRENTSPK | Melanoma, ovarian, gastric, pancreatic [96] and breast carcinomas | Epithelial cells | Kawashima et al. 1999 [66] |
| *hTERT*[c] | A2 | ILAKFLHWL | Lung and ovarian carcinomas – multiple myeloma, melanoma, sarcoma, acute leukemias, non-Hodgkin's lymphomas | Hematopoietic stem cells and progenitors; germinal center cells; basal keratinocytes; gonadal cells; certain proliferating epithelial cells | Vonderheide et al. 1999 [131] |
| *hTRT*[c] | A2 | ILAKFLHWL RLVDDFLLV | Lung, prostate and ovarian carcinomas, multiple myeloma, melanoma, sarcoma, acute leukemias, non-Hodgkin's lymphomas | Circulating B cells; germinal center B cells; thymocytes; CD34+ progenitor hemopoietic cells | Minev et al. 2000 [81] |
| *iCE* | B7 | SPRWWPTCL | RCC | Kidney, colon, small intestine, liver, heart, pituitary gland, adrenal gland, prostate, stomach | Ronsin et al. 1999 [102] |
| *MUC1* | A11 | STAPPAHGV | Breast and ovarian carcinomas, multiple myeloma, B-cell lymphoma | None[d] | Domenech et al. 1995 [31] |
| *MUC1* | A2 | STAPPVHNV | Breast and ovarian carcinoma, multiple myeloma, B-cell lymphoma | None[d] | Brossart et al. 1999 [11] |
| *MUC2* | A2 | LLNQLQVNL MLWGWREHV | Ovary, pancreas and breast mucinous tumors, colon carcinoma of non-mucinous type | Colon, small intestine, bronchus, cervix and gall bladder | Böhm et al. 1998 [7] |
| *PRAME* | A24 | LYVDSLFFL | Melanoma, H/N and lung SCC, NSCLC [122], RCC, adenocarcinoma, sarcoma, leukemias [122] | Testis, endometrium, ovary, adrenals, kidney, brain, skin | Ikeda et al. 1997 [53] |
| *P15* | A24 | AYGLDFYIL | Melanoma | Testis, spleen, thymus, liver, kidney, adrenal tissue, lung tissue, retinal tissue | Robbins et al. 1995 [97] |
| *RU1* | B51 | VPYGSFKHV | Melanoma, renal and bladder carcinomas | Testis, kidney, heart, skin, brain, ovary, liver, lung, lymphocytes, thymus, fibroblasts | Morel et al. 2000 [83] |
| *RU2* | B7 | LPRWPPPQL | Melanoma, sarcomas leukemia – brain, esophageal and H/N tumors – renal, colon, thyroid, mammary, bladder, prostatic and lung carcinomas | Testis, kidney, liver, urinary bladder | Van den Eynde et al. 1999 [124] |
| *SART-1* | A24 | EYRGFTQDF | Esophageal, H/N and lung SCC – adenocarcinoma, uterine cancer | Testis, fetal liver | Kikuchi et al. 1999 [67] |
| *SART-1* | A*2601 | KGSGKMKTE | Esophageal, H/N and lung SCC, adenocarcinoma, uterine cancer | Testis, fetal liver | Shichijo et al. 1998 [107] |
| *SART-3* | A24 | VYDYNCHVDL AYIDFEMKI | H/N, esophageal and lung SCC, adenocarcinoma, leukemia, melanoma | Lymphoid cells, fibroblasts, testis, fetal liver | Yang et al. 1999 [139] |
| *WT1* | A2 | RMFPNAPYL | Gastric, colon, lung, breast, ovary, uterine, thyroid and hepatocellular carcinomas – leukemia (including AML, ALL and CML) | Kidney, ovary, testis, spleen | Oka et al. 2000 [90] |

[a] CAP-1 is an alternative name of this peptide
[b] Tissue distribution among tumors as described in the given references when different from the paper first reporting the sequence of the epitope
[c] Telomerase is expressed in most human tumors: those listed were shown to be susceptible to lysis by cytotoxic T lymphocytes
[d] All epithelial tissues express mucin-like hyperglycosylated molecules

Table 4 Class I HLA-restricted tumor-specific antigens, including both unique (CDK-4, MUM-1, MUM-2, β-catenin, HLA-A2-R170I, ELF2 m, myosin-m, caspase-8, KIAA0205, HSP70-2m) and shared (CAMEL, TRP-2/INT2, GnT-V, G 250) antigens

| Gene | HLA allele | Peptide epitope | Tissue distribution | | Reference |
|---|---|---|---|---|---|
| | | | Tumors | Normal tissues | |
| *AFP* | A2 | GVALQTMKQ | Hepatocellular carcinoma | Fetal liver | Butterfield et al. 1999 [12] |
| *β-Catenin/m* | A24 | SYLDSGIHF | Melanoma | None | Robbins et al. 1996 [98] |
| *Caspase-8/m* | B35 | FPSDSWCYF | H/N tumors | None | Mandruzzato et al. 1997 [78] |
| *CDK-4/m* | A2 | ACDPHSGHFV | Melanoma | None | Wölfel et al. 1995 [138] |
| *ELF2 M* | A68 | ETVSEQSNV | Lung SCC | None | Hogan et al. 1998 [50] |
| *GnT-V* | A2 | VLPDVFIRC(V)[a] | Melanoma, brain tumors, sarcoma | Breast and brain (low expression) | Guilloux et al. 1996 [45] |
| *G250* | A2 | HLSTAFARV | RCC, colon, ovarian and cervical carcinomas | None | Vissers et al. 1999 [129] |
| *HSP70-2M* | A2 | SLFEGIDIY | RCC, melanoma, neuroblastoma | None | Gaudin et al. 1999 [39] |
| *HA-A*0201-R170I* | A2 | CVEWLRIYLENGK | RCC | None | Brändle et al. 1996 [9] |
| *HST-2* | A31 | YSWMDISCWI | Gastric signet cell carcinoma | None | Suzuki et al. 1999 [109] |
| *KIAA0205* | B44*03 | AEPINIQTV | Bladder cancer | None | Gueguen et al. 1998 [44] |
| *MUM-1* | B44 | EEKLIVVLF | Melanoma | None | Coulie et al. 1995 [23] |
| *MUM-2* | B44 | SELFRSGLDY | Melanoma | None | Chiari et al. 1999 [19] |
| *MUM-2* | Cw6 | FRSGLDSYV | Melanoma | None | Chiari et al. 1999 [19] |
| *MUM-3* | A28 | EAFIQPITR | Melanoma | None | Baurain et al. 2000 [4] |
| *Myosin/m* | A3 | KINKNPKYK | Melanoma | None | Zorn and Hercend, 1999a [146] |
| *RAGE* | B7 | SPSSNRIRNT | Melanoma, sarcomas, mesotheliomas, H/N tumors, bladder, renal, colon and mammary carcinomas | Retina only | Gaugler et al. 1996 [41] |
| *SART-2* | A24 | DYSARWNEI | H/N and lung SCC, lung adenocarcinoma, RCC, melanoma, brain tumors, esophageal and uterine cancers | None | Nakao et al. 2000 [85] |
| | | AYDFLYNYL | | | |
| | | SYTRLFLIL | | | |
| *TRP-2/INT2* | A68 | EVISCKLIKR | Melanoma | None | Lupetti et al. 1998 [76] |
| *707-AP* | A2 | RVAALARDA | Melanoma | None[b] | Morioka et al. 1995 [84] |

[a] VLPDVFIRC(V) = nonamer and decamer peptides are both recognized by CTLs
[b] This antigen is not expressed in normal cells but, as the tissue of the testis was not tested, it will not become clear to which category the antigen may belong until more information is available

In mouse models unique antigens have been shown to be more immunogenic than the other groups of shared antigens [32]; since unique antigens are responsible for the rejection of tumor transplants in mice, they have been defined as tumor-specific transplantation antigens (TSTA). The unique antigens are the most specific targets for immunotherapy, but this potential advantage must be balanced against the almost total impossibility of their clinical use, as they can induce an immune response only against the original tumor on which they were found.

Other tumor-specific but shared antigens have been described which are generated by alteration in splicing mechanisms and which occur in tumor but not in normal cells, as in the case of TRP-2/INT2 [76].

Group 5: Class II HLA-restricted antigens (Table 5)

Stimulation of the CD4+ T helper cells by tumor antigens is considered to be impaired or absent in cancer patients and this may be the reason of an insufficient immune response to tumors. Therefore the identification of tumor antigen epitopes recognized by such lymphocytes is a crucial step in the long sought improvement of antitumor immune response that may result into clinical efficacy. The first epitope presented by a class II HLA and capable of provoking a CD4+ T-cell response was identified in 1994 in melanoma tyrosinase [117]. Then a gap of 4 years followed during which only one additional epitope was characterized [118], before other genes encoding class II-restricted peptides were discovered. However, as the technical and methodological approaches for identifying CD4+ T-cell epitopes of tumor antigens have become available, an exponential increase in reporting such epitopes has been seen. In fact, since 1998 as many as 27 new class II HLA-restricted epitopes from 14 antigens have been molecularly identified using, am ng oth rs, li-cDNA fusion libraries [135], immunized transgenic mice [145] and biochemical approaches [96].

**Table 5** Class II HLA-restricted antigens

| Gene | HLA allele | Peptide epitope | Tissue distribution | | Reference |
|---|---|---|---|---|---|
| | | | Tumors | Normal tissues | |
| Epitopes from normal protein antigens | | | | | |
| *Annexin II* | DRB*0401 | DVPKWISIM-TERSVPH | Melanoma | Not done | Li et al. 1998 [73] |
| *Gp100* | DRB1*0401 | WNRQLYPE-WTEAQRLD | Melanoma | Melanocytes | Li et al. 1998 [73] |
| *MAGE-1, -2, -3, -6* | DRB*1301 DRB*1302 | LLKYRAREP-VTKAE | Melanoma, lung and breast carcinomas, H/N SCC | Testis, placenta | Chaux et al. 1999a [16] |
| *MAGE-3* | DR*1101 | TSYVKVLHHM-VKISG | Melanoma, lung and breast carcinomas, H/N SCC | Testis, placenta | Manici et al. 1999 [79] |
| *MAGE-3* | DRB*1301 DRB*1302 | AELVHFLLLK-YRAR | Melanoma, lung and breast carcinomas, H/N SCC | Testis, placenta | Chaux et al. 1999b [17] |
| *MART-1/Melan-A* | DRB1*0401 | RNGYRALMDKS-LHVGTQCALTRR | Melanoma | Melanocytes | Zarour et al. 2000 [143] |
| *MUC1* | DR3 | PGSTAPPAHGVT | Breast and ovarian cancers, multiple myeloma, B-cell lymphoma | None[a] | Hiltbold et al. 1998 [49] |
| *NY-ESO-1* | DRB4*0101 | VLLKEFTVSG | Melanoma, B-lymphoma, hepatoma [18][b], sarcoma, H/N tumors, – bladder, lung, prostate, ovarian, thyroid and breast carcinomas | Testis | Zeng et al. 2000 [145] |
| *NY-ESO-1* | DRB4*0101–0103 | PLPVPGVLLK-EFTVSGNI<br>VLLKEFTVSG-NILTIRLT<br>AADHRQLQL-SISSCLQQL | B-lymphoma, melanoma, sarcoma, H/N tumors, hepatoma [18] – bladder, lung, prostate, ovarian, thyroid and breast carcinomas | Testis | Jäger et al. 2000 [55] |
| *PSA* | DR4 | ILLGRMSLFM-PEDTG<br>SLFHPEDTGQVFQ<br>QVFQVSHSFPHPLYD<br>NDLMLLRLSEPAELT<br>KKLQCVQLHVISM<br>GVLQGITSMGSEPCA | Melanoma | Melanocytes | Corman et al. 1998 [20] |
| *Tyrosinase* | DRB1*0401 | QNILLSNAPLGPQFP<br>DYSYLQDSDPD-SFQD<br>SYLQDSDPDSFQD | Melanoma | Melanocytes | Topalian et al. 1994 [117], Topalian et al. 1996 [118] |
| *Tyrosinase* | DRB1*1501 | RHRPLQEVYP-EANAPIGHNRE | Melanoma | Melanocytes | Kobayashi et al. 1998a [69] |
| *Tyrosinase* | DRB1*0405 | EIWRDIDFAHE | Melanoma | Melanocytes | Kobayashi et al. 1998b [70] |
| Epitopes from mutated protein antigens | | | | | |
| *HPV-E7* | DR*0401<br>DR*0407 | LFMDTLSFVCPLC<br>LFMDSLNFVCPWC | Cervical carcinoma | None | Höhn et al. 1999 [51] |
| *CDC27/m* | DRB1*0401 | FSWAMDLDPKGA | Melanoma | None | Wang et al. 1999a [135] |
| *TPI/m* | DRB1*0101 | GELIGILNAAKVPAD | Melanoma | None | Pieper et al. 1999 [96] |

[a] All epithelial tissues express highly glycosilated mucins whereas tumor cells often show hypoglycosilated mucins with a normal protein sequence

[b] Tissue distribution among tumors as described in the given references when different from the paper first reporting the sequence of the epitope

It is of note that even class II-restricted antigens include a subgroup of mutated proteins which, therefore, represent truly tumor-specific antigens.

## Group 6: Fusion proteins (Table 6)

In several malignancies, particularly in some forms of leukemia, the molecular mechanism of carcinogenesis involves translocation of chromosomes which results in fusion of distant genes. This often causes the synthesis of fusion proteins which characterize each type of disease (e.g., bcr-abl and pml-RARα in CML and APL, respectively) and generate new epitopes that can be recognized by T cells, either CD8+ or CD4+ in class I or class II HLA restriction, respectively. Although these epitopes appear to be weakly immunogenic in leukemia patients [28], some of these peptides or proteins

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Table 6 Epitopes derived from fusion proteins (fusion proteins are never found in normal tissues)

| Gene | HLA allele | Peptide epitope | Tissue distribution among tumors | Reference |
|---|---|---|---|---|
| HLA class I restricted epitopes | | | | |
| *bcr-abl*[a] | A2 | FMVELVEGA<br>KLSEQESLL<br>MLTNSCVKL | CML | Buzyn et al. 1997 [13] |
| *bcr-abl p210(b3a2)* | A2 | SSKALQRPV | CML | Yotnda et al. 1998a [141] |
| *bcr-abl (b3a2)* | A3 | ATGFKQSSK<br>KQSSKALQR | CML | Greco et al. 1996 [43] |
| *bcr-abl p210 (b3a2)* | A3, A11 | HSATGFKQSSK | CML | Bocchia et al. 1996 [5] |
| *bcr-abl p210(b3a2)* | A3 | KQSSKALQR | CML | Norbury et al. 2000 [87] |
| *bcr-abl p210(b3a2)* | B8 | GFKQSSKAL | CML | Norbury et al. 2000 [87] |
| *ETV6/AML* | A2 | RIAECILGM | ALL | Yotnda et al. 1998b [142] |
| HLA class II restricted epitopes | | | | |
| *bcr-abl p190 (e1a2)* | DRB1*1501 | EGAFHGDAEALQRPVAS | ALL | Tanaka et al. 2000 [112] |
| *bcr-abl p210 (b2a2)* | DRB5*0101 | IPLTINKEEALQRPVAS | CML | ten Bosch et al. 1999 [116] |
| *bcr-abl p210 (b3a2)* | DRB1*0401 | ATGFKQSSKALQRPVAS | CML | ten Bosch et al. 1996 [115] |
| *bcr-abl p210 (b3a2)* | DRB1*1501 | ATGFKQSSKALQRPVAS | CML | ten Bosch et al. 1996 [115] |
| *bcr-abl (b3a2)* | DRB1*0901 | ATGFKQSSKALQRPVAS | CML | Yasukawa et al. 1998 [140] |
| *bcr-abl (b3a2)* | DRB1*1101 | LIVVIVHSATGFKQSS-<br>KALQRPVA | CML | Pawelec et al. 1996 [93] |
| *bcr-abl (b3a2)* | DR11 | IVHSATGFKQSSKALQRP-<br>VASDFEP | CML | Bocchia et al. 1996 [5] |
| *Dek-cain* | DRB4*0103 | TMKQICKKEIRRLHQY | AML | Ohminami et al. 1999 [88] |
| *LDLR/FUT* | DRB1*0101 | GGAPPVTWRRAPAPG<br>WRRAPAPGAKAMAPG | Melanoma | Wang et al. 1999b [132] |
| *Pml/RARα* | DR11 | NSNHVASGA-<br>GEAAIETQSSSSEEIV [28] | APL | Gambacorti-Passerini et al. 1993 [38] |
| *p190 minor bcr-abl (e1a2)* | DRB1*1501 | EGAFHGDAEALQRPVAS | AML | Tanaka et al. 2000 [112] |
| *TEL/AML1* | DP5, DP17 | IGRIAECILGMNPSR | AML | Yun et al. 1999 [143] |

[a]These bcr-abl epitopes are not true fusion proteins generated-epitopes, because they derive from outside the bcr-abl junction

Table 7 Frequency of epitopes recognized by a given HLA allele

| Antigen | No. of epitopes | HLA-A | HLA-B | HLA-C |
|---|---|---|---|---|
| MAGE-1, -2, -3, -4, -6, -10, -12 | 24 | 13 (54%) | 7 (29% | 4 (17%) |
| GAGE-1, -2, -3, -4, -5, -6, -7B, -8 | 8 | 5 (62.5%) | 0 | 3 (37.5%) |
| MART-1 | 6 | 4 (67%) | 2 (33%) | 0 |
| Gp100 | 12 | 11 (92%) | 0 | 1 (8%) |
| Tyrosinase | 6 | 5 (83%) | 1 (17%) | 0 |

can nevertheless be used to pulse dendritic cells for vaccination.

### Frequency of epitopes recognized by a given HLA allele (Table 7)

In Table 7 we have summarized, for those antigens from which a high number of epitopes have been described (e.g., CT and differentiation antigens of melanoma) the distribution of epitopes recognized in the context of different HLA loci. This table shows that the majority of epitopes are seen as restricted by HLA-A in all the three groups of antigens considered. Whether this reflects a bias caused by the fact that most of the studies have been carried out with HLA-A-restricted T cells or is mediated by the immun dominant rol of the HLA locus in recognition of tumor antigens remains to be established.

## Conclusions

Several excellent and timely reviews on tumor antigens have been published periodically during the past few years [8, 63, 100]. However, to our knowledge a comprehensive list of all available tumor antigens and their epitopes and HLA restriction has never been reported, despite the fact that the features of each antigen can be easily found in data bases. We hope that our work may be of interest for many tumor immunologists and students. Needless to say, we may have inadvertently missed information on some antigens despite our careful scrutiny of the published literature; therefore, we will be grateful to any readers who provide us with any missing information. We now plan to update these tables bi-m nthly in order to keep our data base as informative as possible. The antigen list can also be found at the INT website (www.istitutotum ri.mi.it).

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